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Effect of Dimethyl Sulfoxide on Cell Cycle Synchronization of *in vitro* Cultured Monkey (*Maccaca fascicularis)* Ear Skin Fibroblasts

Chang-Long Xu, Zu-Xing Qin, Ming-Ming Liang, Huan-Hua Chen, Li Yang, Xi Luo, Jun Luo, Hui-Yan Xu, Jing-Hui Cui, Sheng-Sheng Lu and Ke-Huan Lu
State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Animal Reproduction Institute, Guangxi University, Nanning, 530004 Guangxi, China

Abstract: Arrest of cells in the G0/G1 cell cycle phase is desired for somatic cell nuclear transfer procedures. This study determined the arresting effects of 0-2% Dimethyl Sulfoxide (DMSO) for 4-24 h on cell cycle stages of *Maccaca fascicularis* ear-derived fibroblasts. The effects of DMSO on apoptosis of these cells was also investigated. Cells were obtained from the ear of a 4 years old male monkey. Analysis of cell cycle distribution by fluorescence-activated cell sorting showed that 67.52, 14.37 and 18.11% of normally cycling cells were at G0/G1, S, G2/M phases, respectively. In the groups with 4 h DMSO treatments, cell cycle synchronization in G0/G1 phase for treatment with 1.5 and 2.0% DMSO (82.86 and 86.31%) was significantly higher than in other groups (0.5% for 78.84% and 1.0% for 78.65%) or the control group (67.52%). With 24 h treatments, the proportion of cells in G0/G1 was higher with 1% (90.45%), 1.5% (91.57%) and 2.0% DMSO (98.68%) than with 0.5% DMSO (78.22%) or the control group (70.33%). Under normal culture conditions, 4.63% of cells underwent apoptosis. Treatment with 1.5% DMSO, 2% DMSO for 4 h and 1% DMSO, 1.5% DMSO, 2% DMSO for 24 h resulted in apoptosis in 10.52, 12.75, 10.42, 12.75 and 17.07% of cells, respectively. In conclusion, the use of DMSO is suitable for cell cycle synchronization because it arrests cells at the G0/G1 phase but it also induces a high level of apoptosis, especially after 24 h for cultures treated with 2% DMSO.

Key words: Cell cycle synchronization, G0/G1 phase, monkey, DMSO, nuclear transfer

INTRODUCTION

Since, the revolutionary study of Wilmut *et al*. (1997) reporting the birth of a cloned lamb derived from a somatic cell, many animals have been successfully obtained by Nuclear Transfer (NT) including mice (Wakayama *et al*., 1998), cattle (Kato *et al*., 1998), goat (Baguisi *et al*., 1999) pigs (Polejaeva *et al*., 2000), cat (Shin *et al*., 2002), rabbit (Chesne *et al*., 2002), horse (Galli *et al*., 2003), mule (Woods *et al*., 2003), rat (Zhou *et al*., 2003), ferret (Li *et al*., 2006), wolf (Kim *et al*., 2007) and buffalo (Shi *et al*., 2007), although the success rate is low (≤3% Cardoso *et al*., 1993; Wilmut *et al*., 1997; Wakayama *et al*., 1998). However, with non-human primates, Nuclear Transfer (NT) of embryonic cells has only resulted in live-births in rhesus monkey (Meng *et al*., 1997) and it seems possible that the biological and technical factors that affect the cloning efficiency differ from species to species.

The inefficiency of cloning may be the consequence of multiple factors, especially the cell cycle phase of the donor nucleus and the recipient cytoplasm (Campbell *et al*., 1996; Wolf *et al*., 1998). Developmental rates of reconstructed embryos are higher when donor cells are arrested at the G0/G1 stage of the cell cycle (Wells *et al*., 1997) because this avoids redundant replication of DNA and the genome is reprogrammed (Campbell, 1999; William *et al*., 2001). Thus, this indicates synchronization of the cell cycle stages in the G0/G1 phase as nuclear donors for nuclear transplantation.

Many methods have been used for synchronization of donor cells at the G1 or G0 phase including serum starvation, contact inhibition and/or chemical treatments. Generally, either contact inhibition (Boquest *et al*., 1999; Holker *et al*., 2005) and serum starvation (Cho *et al*., 2002; Wilmut *et al*., 1997) are the most frequently used methods. In addition, chemical inhibitors such as roscovitine (Gibbons *et al*., 2002; Sun *et al*., 2008) or Cycloheximide (CHX) (Goissis *et al*., 2007) have been used to synchronize cells at the G1 or G0 phase of the cycle. Recently, the effects of Dimethyl Sulfoxide (DMSO) on cell cycle arrest have been investigated in several animals including goral (Hashem *et al*., 2006), Siberian

Corresponding Author: Sheng-Sheng Lu, State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Animal Reproduction Institute, Guangxi University, Nanning, 530004 Guangxi, China

tiger (Hashem *et al*., 2006), brown bear (Caamano *et al*., 2008), dog (Koo *et al*., 2009) and fish (Choresca *et al*., 2009).

However, only a few studies have been conducted on the control of cell cycle stages in monkey and there is little information available on the effects of chemical inhibitors. Therefore, as part of a general goal of improving the efficiency of cloning in monkeys, the present study has evaluated the effects of various concentrations and exposure times of DMSO on cell cycle synchronization at the G0/G1 phase.

MATERIALS AND METHODS

Chemicals: All chemicals were purchased from the Sigma Chemical Company (St. Louis, MO) unless otherwise stated.

Cell culture: All animal procedures were approved in advance by the Institutional Animal Care and Use Committee of State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, Animal Reproduction Institute, Guangxi University. Ear tissue was obtained from a 4 years old male *Maccaca fascicularis* monkey. Small pieces of ear skin tissue were washed 3 times and minced in Ca^{2+} and Mg^{2+} free Phosphatephosphate-Buffered Saline (PBS) containing antibiotics (penicillin G 250 IU mL^{-1} and streptomycin 250 μg mL^{-1}). The minced tissues were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) supplemented with 10% (v/v) Fetal Bovine Serum (FBS; Invitrogen), 1 mM glutamine (Invitrogen), 25 mM $NaHCO_3$ and 1% (v/v) minimal essential medium non-essential amino acid solution (Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Monolayer cells with fibroblast-like morphology were passaged in DMEM with 10% FBS and harvested with PBS containing 0.25% (w/v) trypsin and 0.2% (w/v) EDTA when the culture to 100% confluence. The fibroblast cells were then passaged two more times before being frozen in DMEM with 10% FBS and 10% DMSO and stored in liquid nitrogen. Cells were thawed at 37°C and cultured in DMEM with 10% FBS. Three to eight passages were used for this experiment.

Treatment of cells: For the cycling group, cells were at passage 3-8 and 60-80% confluence. The effects of different concentrations (0, 0.5, 1.0, 1.5 and 2%) of DMSO for 4 or 24 h in inducing cell cycle arrest at the G0/G1 phase were evaluated by treating cells that had reached 60-80% confluence. The cells were then washed, harvested and fixed for further analysis.

Cell cycle analysis: Cells were digested with 0.25% trypsin/EDTA and resuspended in DMEM at a concentration of 1×10^6 cells/tube. Cell cycle was analyzed by FACS (Fluorescence-Activated Cell Sorting according to Hashem *et al*. (2006). In brief, the cell suspension was first centrifuged for 5 min at 1,200 rpm at 4°C. Cells were fixed by dropwise addition of 0.7 mL cold ethanol (70%) into a tube containing 0.3 mL of cell suspension in PBS with gentle vortexing. The fixed cells were then maintained at 4°C for 48 h prior to further analysis. The fixed cells were washed in cold PBS to remove ethanol, resuspended in 0.25 mL of PBS containing 5 uL of 10 mg mL^{-1} Rnase and then incubated for 1 h at 37°C. After 1 h of incubation, cells were stained by adding 10 μL of 1 mg mL^{-1} propidium iodide. Samples were analysed using FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA). Fluorescence data were obtained from 10,000 cells from each sample. The percentages of cells existing within the various phases of the cell cycle were automatically calculated by the Modfit LT 3.0 Software (verity software house) using the same algorithm for all the samples.

Analysis of cell apoptosis: The effect of different treatments on cell apoptosis was determined using Hoechst 33342 staining according to Han *et al*. (2006). Cells were collected in a 1.5 mL tube on ice by centrifugation (200×g, 5 min) at 4°C. The cell pellets were re-suspended in 50 μL D-PBS supplemented with 0.01 mg mL^{-1} Hoechst 33342 (Sigma) and stained in the dark for 5 min. Then, microliter drops of suspension were smeared on a slide and observed under a fluorescence microscope (1000x, under oil). Cells with pycnotic nuclei were considered to be apoptotic (Shiota *et al*., 2003; Chen *et al*., 2005). Four to six fields of each slide were examined and 200 cells on each slide were analyzed to evaluate the degree of apoptosis.

Data analysis: Three replicates were conducted for each treatment. Statistical analyses were carried out by one-way Analysis of Variance (ANOVA). Differences between treatment groups were performed using the Duncan multiple comparison test. The software used was SPSS (Statistical Package for Social Sciences, Version 11.5, SPSS, Inc., Chicago, IL). Data are expressed as means±SEM and $p<0.05$ is considered significant.

RESULTS

As shown in Table 1, 67.52, 14.37 and 18.11% of cycling cells (controls) were in the G0/G1, S and G2/M phases, respectively. The effects of different

Table 1: Effect of different levels of DMSO on the synchronization of cell cycles of *Maccaca fascicularis* ear skin fibroblasts after 4 h treatment

	Cells at different cell cycle (mean %±SEM)		
Treatments	G0/G1	S	G2/M
0[#]	67.52±6.05[a]	14.37±4.68[a]	18.11±9.97[a]
0.5%	77.84±0.49[ab]	10.02±1.79[a]	12.15±0.32[a]
1.0%	78.65±0.21[ab]	10.32±0.37[a]	11.03±0.43[a]
1.5%	82.86±1.73[b]	6.25±1.91[a]	10.89±3.43[a]
2%	86.31±6.55[b]	6.12±2.85[a]	7.57±3.72[a]

Values within columns with different superscripts are significantly different ($p<0.05$); [#]Cycling cells without DMSO treatment as control group

Table 2: Effect of different levels of DMSO on the synchronization of cell cycles of *Maccaca fascicularis* ear skin fibroblasts treated for 24 h

	Cells at different cell cycle (mean %±SEM)		
Treatments	0/G1	S	G2/M
Control[#]	70.33±3.15[a]	10.76±5.02[a]	18.91±7.33[a]
0.5%	78.22±0.83[a]	14.33±0.47[a]	7.45±0.52[ab]
1.0%	90.45±5.72[b]	7.99±6.02[ab]	1.55±1.55[b]
1.5%	91.57±0.23[b]	7.18±0.29[ab]	1.25±0.41[b]
2%	98.68±0.18[b]	1.02±0.14[b]	0.30±0.17[b]

Values within columns with different superscripts are significantly different ($p<0.05$); [#]Cycling cells without DMSO treatment

concentrations (0, 0.5, 1.0, 1.5 and 2%) of DMSO for 4 h in inducing cell cycle arrest at the G0/G1 phase were analyzed by FACS and the results demonstrate that cell cycle synchronization in the G0/G1 phase for cells treated with 1.5 and 2.0% DMSO for 4 h (82.86 and 86.31%) was significantly higher ($p<0.05$) than that for other groups treated for 4 h (0.5% for 78.84%, 1.0% for 78.65%) or the control group (67.52%). There was no significant difference ($p>0.05$) in the percentage of S and G2/M phase cells in the control group and the groups with different levels of DMSO treatment for 4 h.

The effects of different concentrations (0, 0.5, 1.0, 1.5 and 2%) of DMSO for 24 h on cell cycle arrest at the G0/G1 phase are shown in Table 2. The proportions of cells in the G0/G1 phase were higher ($p<0.05$) for treatment with 1% (90.45%), 1.5% (91.57%) and 2.0% DMSO (98.68%) than for 0.5% DMSO (78.22%) and the control group (70.33%). The percentage of S phase cells in the control group (10.76%) was significantly higher ($p<0.05$) than in those treated with 2% DMSO (1.02%) but the DMSO (0.5, 1.0, 1.5 and 2%) treated groups showed significantly lower ($p<0.05$) percentage at the G2/M phase than the control group (7.45, 1.55, 1.25 and 0.30 vs. 18.91%).

The effects of DMSO on apoptosis are shown in Fig. 1. Under normal culture conditions, 4.63% of cells underwent apoptosis but DMSO increased cell apoptosis at all concentrations investigated and 1.5% DMSO, 2% DMSO for 4 h and 1% DMSO, 1.5% DMSO, 2% DMSO for 24 h caused apoptosis in 10.52, 12.75, 10.42, 12.75 and 17.07% of cells, respectively.

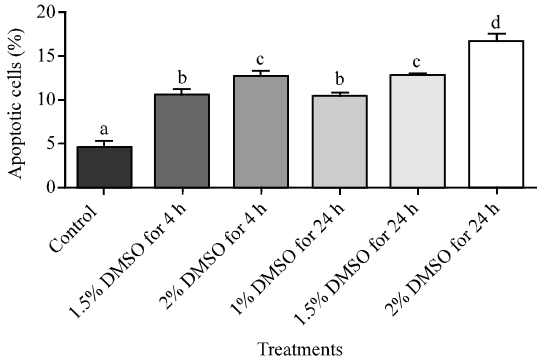

Fig. 1: The effect of different concentrations and exposure times of DMSO on cell apoptosis of *Maccaca fascicularis* ear skin fibroblasts stained with Hoechst 33342. Values with different superscripts (a-d) are significantly different ($p<0.05$). Control, 0% DMSO at 48 h

DISCUSSION

Cell cycle coordination between nuclear donor and recipient cells is considered to be one of the crucial factors for successful cloning because of the need to maintain the correct ploidy of embryos at the end of the first cell cycle (Campbell *et al*., 1996; Wolf *et al*., 1998). For successful reprogramming of the donor nucleus by nuclear transfer, the donor nuclei must be in the G0 or G1 phase when transferred to metaphase II recipient oocytes (Hayes *et al*., 2005). To obtain somatic cells in the G0 or G1 stage of the cell cycle, several methods have been used (Kubota *et al*., 2000; Kues *et al*., 2000; Gibbons *et al*., 2002; Zou *et al*., 2002; Gomez *et al*., 2003; Han *et al*., 2003; Liu *et al*., 2004; Hayes *et al*., 2005). In the present study, researchers analysed the effect of different levels of DMSO at different exposure times on cell cycle synchronization in *Maccaca fascicularis* ear skin fibroblasts. Generally, DMSO is regarded as a cryoprotectant and liphophilic solvent for cells *in vitro* (Kudo *et al*., 2002), although it also induces and maintains cell differentiation in human leukemic cells and in human and rat hepatocytes (Avery and Greve, 2000). The results showed that 4 h treatments with 0.5 or 1% DMSO were less efficient than the control group (Table 1) but 1.5 and 2% DMSO induced higher cell cycle synchronization in the G0/G1 phase. To achieve a higher percentage of G0/G1 cells, a longer duration of treatment was considered. The results in Table 2 show that the percentage of cells at the G0/G1 phase increased significantly after treatment for 24 h with 1.0, 1.5 or 2% DMSO. In addition, 0.5% DMSO was equally effective for synchronization of cells in the G0/G1 phase for 4 and 24 h treatments. Other

researchers have reported similar synchronization rates for cells of adult goral skin fibroblasts at G0/G1 phases for 24 and 4 h treatments at 0.5% DMSO (Hashem *et al*., 2006).

On the other hand, Choresca *et al*. (2009) reported that treatment with 0.5, 1.0 and 1.5% DMSO decreased the proportion of G0/G1 cells at 24 h of treatment in synchronizing goldfish caudal fin-derived fibroblast cells. Koo *et al*. (2009) obtained a similar result with canine fibroblasts and reported that treatment with 0.5% DMSO decreased the proportion of G0/G1 cells at 24 h. This is possibly because of the expression of cyclins A, B and E, since this enhances progression from late G1 to the G2/M phase and thus decreases the proportions of G0/G1 cells. In the study, DMSO significantly increased the percentage of cells in the G0/G1 phase in different groups (1.5 and 2% DMSO for 4 h; 1, 1.5 and 2% DMSO for 24 h) whereas the percentage of cells in the S and G2/M stages decreased in a dose dependent fashion in the 24 h treatment groups. Others have reported an increased percentage of goral skin fibroblast cells in the G0/G1 stage after culturing with 0.5 and 1% DMSO for 24 h (Hashem *et al*., 2006) no effect of DMSO on the cell cycle stages of cultured skin fibroblasts of the Siberian tiger (Hashem *et al*., 2006) and no differences between control and cells treated with DMSO for 72 h in porcine cultured mammary cells (Prather *et al*., 1999). These apparently contradictory results may be the consequence of using different concentration of DMSO, differences in cell types, animal species or species sensitivity to this chemical.

For 4 h treatment groups, DMSO concentrations of 1.5 and 2% significantly induced the percentage of cells in the G0/G1 phase in Crab-eating Macaque ear skin fibroblasts (82.86 and 86.31%) which is similar to the report in mammalian species. Hashem *et al*. (2006) found 74.8 and 75.2% G0/G1 phase percentages in cultured goral adult skin fibroblasts treated with 0.5 and 1.0% DMSO for 4 h and 79.1, 79.1 and 79.0% G0/G1 phase percentages were observed in brown bear fibroblasts treated with 1, 2 and 3% DMSO for 48 h (Caamano *et al*., 2008).

With 24 h treatments (Table 2), the proportion of cells in G0/G1 was higher ($p<0.05$) with 1% (90.45%), 1.5% (91.57%) and 2.0% DMSO (98.68%) than with 0.5% DMSO (78.22%) and the control group (70.33%). Choresca *et al*. (2009) found a significant percentage increase in the number of cells at the G0/G1 phase when goldfish caudal fin-derived fibroblasts were treated with 1% DMSO for 48 h compared to 24 h. Also, in the study of the cell cycle of canine ear fibroblasts (Koo *et al*., 2009), the percentage of cells at the G0/G1 stage following DMSO treatment for 72 h (76.1%) was higher than for 24 h (67.6%) or 48 h (70.5%).

In the present study, reserachers found a higher percentage of apoptotic cells in all DMSO treatment groups (Fig. 1) and this increased with both level and duration of exposure to DMSO and the percentage was highest (17.07%) for 2% DMSO and 24 h. High levels of apoptotic cells were detected by Choresca *et al*. (2009). In goldfish cells treated with 1% DMSO for 48 h. In addition, Hashem *et al*. (2006) speculated that treatment with 2.5% DMSO had a detrimental effect on goral cells, a result which was supported by Caamano *et al*. (2008) who found that brown bear fibroblast cells were sensitive to higher concentrations of DMSO.

CONCLUSION

The effect of DMSO in synchronizing *Maccaca fascicularis* ear skin fibroblast cells in G0/G1 phase was achieved for 24 h with 2% concentration. However, there was also an increase in the percentage of apoptotic cells and we recommend using 1.5% DMSO or 2% DMSO for 4 h and 1% DMSO or 1.5% DMSO for 24 h to obtain more healthy G0/G1 cells. These results, therefore, provide baseline data for such investigations and could have a positive impact on somatic cell nuclear transfer in the monkey.

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