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Effects of Serum Concentration, Synchronization Time and Confluence on the Cell-Cycle Synchronization Efficiency of Goat Fibroblasts

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Abstract

This study aims to evaluate the effect of serum concentration, synchronization time, and confluence degree on the synchronisation efficiency of goat fibroblast cycle. The results indicated that there was no difference in the percentage of nucleated fibroblasts in the G0/G1 stage between serum concentrations of 0.3% and 0.4% (83.89% and 82.69%, respectively, $P > 0.05$) as well as between serum concentrations of 0.2% and 0.5% (76.95% and 75.46%, respectively, $P > 0.05$). The percentage of nucleated fibroblasts in the G0/G1 stage was highest at the concentration of 0.3% and lowest in the control group (83.89% vs. 62.67%, $P < 0.05$). The results of evaluating the influence of synchronization time showed that the percentage of nucleated fibroblasts in the G0/G1 stage was highest in the 72-hour group compared to the 24-hour, 48-hour, 96-hour and 120-hour groups (85.87% vs. 79.35%; 83.71%; 72.37% and 67.85%, respectively, $P < 0.05$). Although the percentage of nucleated fibroblasts at the G0/G1 stage of the 72-hour group was higher than the 48-hour group, this difference was insignificant (85.87% and 83.71%, respectively, $P > 0.05$). The beneficial effect of high confluence was confirmed by the large percentage of nucleated fibroblasts at the G0/G1 stage. The 60% confluency was significantly lower than the 80% and 100% confluency (73.44%, 86.63%, and 87.17%, respectively, $P < 0.05$). The results indicate that the goat fibroblast cycle synchronization is the most effective at the serum concentration of 0.3%, 72 hours of synchronization and 100% confluency.

Keywords

Goat Fibroblast, Cycle Synchronisation, Serum Concentration,

Synchronization Time, Confluence

1. Introduction

The success in creating cloned animals using the somatic cell nuclear transfer (SCNT) technique offers many potential applications in basic research, biomedicine, and agriculture [1]. The success of creating cloned animals using SCNT in some animal species was reported, such as buffalos [2], dogs [3], pigs [4], horses [5], cats [6], and goats [7]. The SCNT technique has many limitations such as success rates remaining very low in all species with an average of only about 1% of reconstructed embryos leading to live offspring, the high of cloned offspring dying late in pregnancy or soon after birth, this is due to respiratory or cardiovascular problems [8]. Some studies reported the successful creation of cloned goats. However, the low efficiency of applying the SCNT technique is the main obstacle to the widespread application of this technique [9].

The low efficiency of the SCNT technique is reflected in the low embryo development rate to the implantation stage and the high miscarriage rate during pregnancy, leading to poor offspring survival. There are many reasons to explain this problem, such as asynchrony between donor and recipient cells [10], abnormal genetic and chromosomal manifestations of donor cells [11], embryonic gene expression altered [12], the high SCNT embryo apoptosis rate [13], in which the synchronization between the cycles of the donor cell nucleus and the recipient oocyte protoplasm is a necessary factor for creating a complete cloned embryo from somatic cell nuclear transfer. Donor cells commonly used for the SCNT to create cloned animal embryos are fibroblasts collected from ear tissue or foetal skin [14].

Some studies suggested that using donor cells with nuclei at G0/G1 stage increases the efficiency of creating cloned embryos using SCNT. To obtain nucleated donor cells at the G0/G1 stage, donor cells are mainly grown in a deprived serum culture environment [15]. The productivity of synchronising the fibroblast cycle using low-serum culture depends on several factors, such as cell cycle culture time, serum concentration in the culture medium, and confluence degree of donor cells before synchronization. Therefore, choosing the synchronization culture time, concentration of the culture medium and confluence degree before synchronization will bring high efficiency in creating cloned animal embryos using the SCNT technique. This study aims to optimise cell-cycle synchronization conditions for goat fibroblasts and to set the stage for creating cloned goat embryos in Vietnam.

2. Material and Methods

All the experimental procedures used in this study were performed in accordance with Vietnam legislation and according to Decision No. 1137/QĐ-BNN-KHCN of the Ministry of Agriculture and Rural Development in Vietnam on

March 28.2023.

2.1. Reagents and Chemicals

All reagents and chemicals used in this study were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Petri dishes used for cell culture are originated from Corning Inc. (Corning, NY, USA).

2.2. Ethics Statement and Ear Tissues Collection

Ear tissues collection was carried out according to the standard animal care in Vietnam as per guidelines from the Vietnam National Institute of Animal Sciences (01/2012). Because there are no specific rules regarding animal welfare in Vietnam, we followed the rules in accordance with Vietnamese Law on Animal Health (2015, https://vanban.chinhphu.vn/default.aspx?pageid=27160&docid=180584) and Vietnamese Law on Animal Husbandry (2018, https://vanban.chinhphu.vn/?pageid=27160&docid=206100). However, these laws do not clearly explain how to use animals in research. Hence, ear tissues collection in our study was conducted according to the guidelines for using animals in research based on EU Directive 2010/63.

2.3. Ear Tissue Collection, Isolation and Culture of Cells

Ear tissues were collected from Saanen goats at Thuc Thiem goat farm, Ba Vi, Hanoi, Vietnam. These ear tissues were washed with 70% alcohol and completely removed fat and hair. These tissue were transferred into tubes containing DPBS supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin and immediately transported to the laboratory within 2 - 3 hours. Ear tissues were washed five times in DPBS, then cut into 1 mm^3 pieces, placed on the bottom of a culture plate containing DMEM and 10% foetal bovine serum, and cultured at 37˚C, 5% CO_2, and saturated air humidity. Change the medium and remove any loose tissue from the bottom of the dish at 24 h post-culture. During the culture, any culture plate infected by bacteria will be removed immediately. When the cells reach 80% - 90% coverage, they will be harvested with 0.25% Trypsin EDTA and continue to be transferred to another culture plate at a ratio of 1:2 or 1:3.

2.4. Evaluation of the Developmental Stage of Fibroblast Nuclei Using Fluorescence-Activated Cell Sorting (FACS)

After synchronization, fibroblasts will be treated with Trypsin EDTA and washed with PBS. Next, mix the cells in PBS to ensure the cells are entirely separate from each other. The cell density is 1×10^6 - 1×10^7 cells/500µl PBS. Incubate fibroblasts in 70% ethanol solution maintained at 4˚C for 2 hours. Fibroblasts can be kept in the refrigerator for 2˚C - 8˚C or placed on ice. After fixation, centrifuge at a speed of 2000 rpm for 5 minutes. Remove alcohol. Dissolve the cells in 5 ml of PBS, wait 60 seconds, and then centrifuge again with the same speed and time. Discard the supernatant. Dissolve the cells in 5 ml of PBS containing Triton-X 100, propidium iodide (PI) dye, containing RNAse A, and in-

cubate for 15 - 20 minutes at 37˚C or 30 - 45 minutes at room temperature. Samples were analysed by a BD FACSCanto II Flow Cytometer (BD Biosciences, NJ, USA) excited by a 488-nm argon laser source. The emitted light is collected by a filter system for the red wave bands.

2.5. Synchronization of the Fibroblast Cycle

Fibroblasts were washed three times in DMEM medium and then transferred to culture in DMEM co-phase medium with serum concentration, synchronization time, and coverage depending on the experiment. Fibroblasts grown in DMEM medium supplemented with 10% serum will used as the control group.

2.6. Experiment Design

Experiment 1: *Evaluation of the effect of serum concentration on the cell-cycle synchronization efficiency of goat fibroblasts*

In this experiment, we synchronised goat fibroblasts at four different serum concentrations: 0.2%, 0.3%, 0.4% and 0.5% with 48-hour synchronisation time and 80% coverage. The experiment was repeated six times.

Experiment 2: *Evaluation of the effect of synchronization time on the efficiency of cell-cycle synchronization of goat fibroblasts*

In this experiment, goat fibroblasts were synchronised at five different synchronization times: 24 hours, 48 hours, 72 hours, 96 hours and 120 hours at the serum concentration selected in experiment 1 and 80% coverage. The experiment was repeated six times.

Evaluation of the effect of confluence degree on the cell-cycle synchronization efficiency of goat fibroblasts

In this experiment, goat fibroblasts were synchronised at three different confluence levels: 60%, 80% and 100% at the serum concentration selected in experiment 1 and synchronization time selected in experiment 2. The experiment was repeated six times.

The efficiency of goat fibroblast cycle synchronisation in three experiments was evaluated based on the ratio of nucleated goat fibroblasts at the G0/G1 stage.

2.7. Data Analysis

All data were expressed as mean ± SEM values and analysed by ANOVA, followed by Tukey's multiple comparisons test, using Graph-Pad Prism software (Version 7.02 for Windows, GraphPad Software, La Jolla, California, USA). To compare two groups, data was analysed by T-test. $P < 0.05$ was defined as significant difference.

3. Results

3.1. Effect of Serum Concentration on the Cell-Cycle Synchronization Efficiency of Goat Fibroblasts

The results of evaluating the influence of serum concentration on the efficiency

of goat fibroblast cycle synchronization are shown in Table 1. The results in Table 1 showed that there is no difference in the percentage of nucleated fibroblasts in the G0/G1 phase of the cell cycle at serum concentrations of 0.3% and 0.4% (83.89% vs82.69%, respectively, $P > 0.05$), serum concentrations of 0.2% and 0.5% (76.95% vs75.46%, respectively, $P > 0.05$). The percentage of nucleated fibroblasts in the G0/G1 phase of the cell cycle was highest at the concentration of 0.3% and lowest in the control group (83.89% and 62.67%, respectively, $P < 0.05$).

3.2. Effect of Synchronization Time on the Cell-Cycle Synchronization Efficiency of Goat Fibroblasts

With the results achieved in experiment 1, in this experiment, we used 0.3% serum in a synchronization medium. The results of evaluating the influence of synchronization time on the efficiency of goat fibroblast cycle synchronization are shown in Table 2.

The results shown in Table 2 show that the synchronization time affects the efficiency of cell cycle synchronization in the G0/G1 phase. The percentage of goat fibroblasts with nuclei at the G0/G1 stage was highest in the 72-hour group when compared with the 24-hour, 48-hour, 96-hour, and 120-hour groups (85.87% vs 79.35%, 83.71%, 72.37% and 67.85%, respectively). Although the percentage of goat fibroblasts with nuclei at the G0/G1 stage in the 72-hour group was higher than in the 48-hour group, this difference was insignificant (85.87% and 83.71%, respectively, $P > 0.05$).

3.3. Effect of Confluence Degree on the Cell-Cycle Synchronization Efficiency of Goat Fibroblasts

With the results achieved in experiment 2, in this experiment, we used goat fibroblasts with 0.3% serum for 72 hours. The results of evaluating the influence of confluence degree on the efficiency of goat fibroblast cycle synchronization are shown in Table 3. The results shown in Table 3 show that confluence degree of goat fibroblasts before synchronization affects the synchronization efficiency of goat fibroblasts. The percentage of goat fibroblasts with nuclei at the G0/G1 stage in the 60% group is significantly lower than the 80% and 100% groups (73.44% vs 86.63%, and 87.17%, respectively, $P < 0.05$).

4. Discussion

This result shows that culturing goat fibroblasts in a deprived serum culture medium increases the percentage of fibroblasts with nuclei at the G0/G1 phase of the cell cycle. Using a FACS system to test the efficiency of goat fibroblast cycle synchronization (Table 1, Figure 1) showed that culturing goat fibroblasts at a concentration of 0.3% or 0.4% was sufficient to produce more than 80% of fibroblasts with nuclei at the G0/G1 phase of the cell cycle.

Deprived serum condition is commonly used to synchronise the fibroblast cycle to the G0/G1 phase [15]. Fibroblasts in culture often require adequate

Table 1. Effect of serum concentration on synchronization efficiency of goat fibroblast cycle.

G0/G1 stage	Serum concentration				
	0.2%	0.3%	0.4%	0.5%	Control
% (Mean ± SE)	76.95^{b} ± 2.19	83.89^{a} ± 1.64	82.69^{a} ± 1.82	75.46^{b} ± 1.74	62.67^{c} ± 1.45

Six replications were performed. Percentage data are shown as mean ± SEM. a, b, c in the same row differ significantly ($P < 0.05$).

Table 2. Effect of synchronization time on the synchronisation efficiency of goat fibroblast cycle.

G0/G1 stage	Culture time				
	24 hours	48 hours	72 hours	96 hours	120 hours
% (Mean ± SE)	79.35^{b} ± 1.82	83.71^{a} ± 1.71	85.87^{a} ± 1.82	72.37^{c} ± 2.06	67.85^{d} ± 1.98

Six replications were performed. Percentage data are shown as mean ± SEM. a, b, c, d in the same row differ significantly ($P < 0.05$).

Table 3. Effect of confluence degree on the synchronization efficiency of goat fibroblast cycle.

G0/G1 stage	Confluence degree		
	60%	80%	100%
% (Mean ± SE)	73.44^{a} ± 2.61	86.63^{b} ± 1.99	87.17^{b} ± 1.42

Six replications were performed. Percentage data are shown as mean ± SEM. a, b in the same row differ significantly ($P < 0.05$).

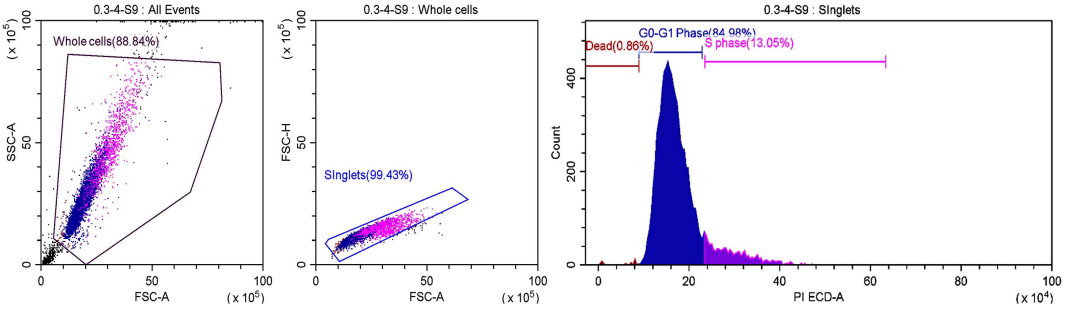

Figure 1. Results of synchronisation of goat fibroblasts at 0.3% serum concentration (48 hours of synchronization, 80% confluency). The resolution of **Figure 1** is 289 × 1017 (pixels).

nutritional requirements to complete their mitosis. Serum is often used for *in vitro* cultures of cell animals. According to Liu *et al.* (2023) [16], the present of serum in the culture media as a source of growth and adhesion factors, hormones, lipids, and minerals for the cell culture. When cells are grown in a low-serum culture environment, mitogens and hormones in the culture environment will be reduced, metabolism will decrease, and the cells will return to a resting state (G0) [17]. According to Iyer *et al.* (1999) [17], serum deprivation during culture leads to the absence of mitotic signals; thus the cells exit from the

cell cycle into the G0 phase rapidly with low metabolic activity. Reducing the serum content in the culture medium causes the degradation of cell proteins, thereby causing the cells to quickly return to the G0 state [18].

The results of our study show that the percentage of goat fibroblasts with nuclei at the G0/G1 stage increased significantly when the time of culturing fibroblasts in serum-deficient conditions was extended to 72 hours (85.87%) (**Table 2**, **Figure 2**). This result is higher than that reported by Kues *et al.* (2000) (78.5%) [15]. However, when increasing the synchronization time to 96 hours and 120 hours, the percentage of fibroblasts with nuclei in G0/G1 tends to decrease (72.37% and 67.85%, respectively). These results are consistent with those reported by Kues *et al.* (2000) [15], Goissis *et al.* (2007) [19], and Sun *et al.* (2008) [20].

In the present study, a synchronization time of more than 72 hours did not increase the percentage of nucleated fibroblasts in the G0/G1 phase of the cell cycle. This result is consistent with the report of Kues *et al.* (2000) [15], Goissis *et al.* (2007) [19] and Sun *et al.* (2008) [20]. Goissis *et al.* (2007) [19] cultured pig fibroblasts in serum deprivation conditions for 24 hours, 48 hours, 72 hours, 96 hours, 120 hours, and 144 hours. Their research results showed that the percentage of fibroblasts with nuclei at G0/G1 was highest in the 48-hour group (86.79%) and tended to gradually decrease in the 72-hour, 96-hour, 120-hour and 144-hour groups (86.09%, 84.27%, 85.45% and 84.18%, respectively). According to Miranda *et al.* (2009) [21], increasing the time of culturing fibroblasts in deprived serum conditions will reduce growth factors, steroids, and some factors necessary for mitotic stimulation and the viability of fibroblasts in deprived serum conditions. This is probably the cause of the phenomenon of nucleated fibroblasts at the G0/G1 stage gradually decreasing when culturing fibroblasts in deprived serum conditions is prolonged.

Results of evaluating the effect of confluence degree on the efficiency of goat fibroblast cycle synchronization show that even though the percentage of goat fibroblasts with nuclei at the G0/G1 stage in the 100% group was higher than the 80% group, this difference was not significant ($P > 0.05$) (**Table 3**, **Figure 3**). These results are similar to those reported by Sun *et al.* (2008) [20], Dalman *et al.* (2010) [22]. According to Sun *et al.* (2008) [20], at synchronizing, bovine fibroblasts with 100% confluency resulted in the percentage of nucleated fibroblasts in the G0/G1 stage reaching 91.5% after synchronization. Dalman *et al.* (2010) [22] showed that the use of full confluency is suitable for cell cycle synchronization goat fibroblasts by serum starvation. Meanwhile, according to Miranda *et al.* (2009) [21], when bovine fibroblasts were synchronized with 60% - 70% confluency, the percentage of fibroblasts with nuclei at the G0/G1 stage reached 84.1%. When the coverage of the bottom of the culture plate by fibroblasts reaches 100%, fibroblasts no longer have any place to adhere, proliferate, and develop. Therefore, some fibroblasts will temporarily stop dividing and stay resting without proliferation. Besides, some fibroblasts also stop dividing due to low serum concentration in the culture medium, so this may be the reason for

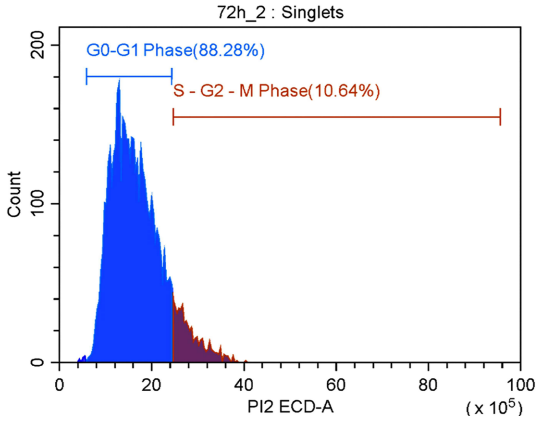

Figure 2. Results of synchronization of goat fibroblasts with 72-hour synchronization time (0.3% serum concentration, 80% confluency). The resolution of **Figure 2** is 520 × 531 (pixels).

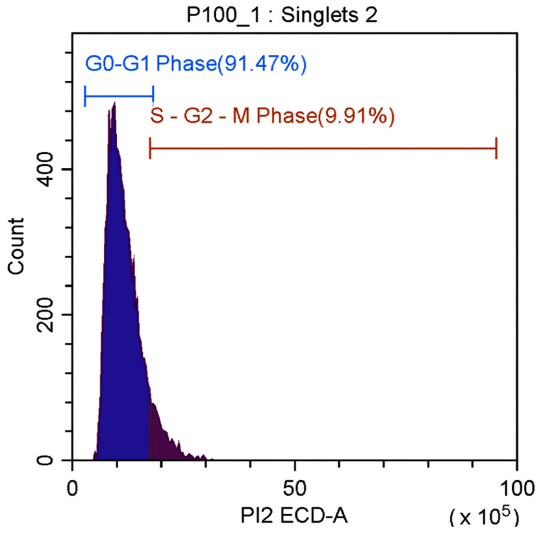

Figure 3. Result of synchronization of goat fibroblasts with 100% confluency (0.3% serum, synchronization time 72 hours). The resolution of **Figure 3** is 500 × 579 (pixels).

the phenomenon of the ratio of nucleated fibroblasts at the G0/G1 stage of the 100% group is higher than the 80% and 60% groups.

These results show that goat fibroblasts were successfully synchronised by using the deprived serum culture method at 0.3% serum concentration, 100% confluency, and 72-hour synchronization time with the rate of goat fibroblasts having nuclei at the G0/G1 stage reaching more than 80%. According to Miranda *et al.* (2009) [21], the rate of fibroblasts with nuclei at the G0/G1 stage of more than 80% is enough to use for nuclear transfer and cloned embryo creation.

5. Conclusion

In this study, our objective was to ascertain the effects of serum concentration,

synchronization time and confluence on the cell cycle synchronization efficiency of goat fibroblasts. Our findings indicate that the goat fibroblast was effectively synchronized by serum deprivation at 0.3% serum concentration, 72 hours of synchronization and 100% confluency.

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Conflicts of Interest

The authors declare no conflicts of interest.

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