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Optimization of Serum Concentration, Synchronization Duration, and Cell Confluence on the Cell-Cycle Synchronization Efficiency in Thanh Chương Buffalo Fibroblasts

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Abstract

This study aimed to optimize serum concentration, synchronization duration, and cell confluence to improve cell-cycle synchronization efficiency in Thanh Chương buffalo fibroblasts. The proportion of nucleated fibroblasts in the G0/G1 phase was the highest at a serum concentration of 0.3% (81.26%) and the lowest in the control group (61.44%), with the difference being statistically significant ($P < 0.05$). No significant differences were observed at serum concentrations of 0.2%, 0.4%, and 0.5% ($P > 0.05$). The proportion of fibroblasts in the G0/G1 phase was significantly higher in the 96-hour and 120-hour synchronization groups compared with the 24-hour, 48-hour, and 72-hour groups ($P < 0.05$). However, no significant difference was detected between the 96-hour and 120-hour groups ($P > 0.05$). Similarly, fibroblasts at 80% and 100% confluence exhibited a significantly higher proportion in the G0/G1 phase compared with those at 60% confluence (86.32% and 85.91% vs 70.56%, respectively; $P < 0.05$). In conclusion, the cell-cycle synchronization efficiency of Thanh Chuong buffalo fibroblasts was enhanced by serum deprivation at a concentration of 0.3%, with a synchronization period of 96 hours and confluency levels exceeding 80%.

Keywords

Buffalo Fibroblast, Cycle Synchronisation, Serum Concentration, Synchronization Duration, Cell Confluence

1. Introduction

Preservation of animal genetic resources constitutes a critical of both genetic improvement programs and biodiversity conservation strategies [1]. Currently, the preservation of livestock genetic resources such as sperm, embryos, somatic cells represents a practical and widely adopted strategy [2]. The successful generation of cloned animals through somatic cell nuclear transfer (SCNT) has opened avenues for diverse applications across fundamental research, biomedical innovation, and agricultural development [3]. Shi *et al.* (2007) [4] reported the first successful production of a swamp buffalo using the SCNT technique. However, the low efficiency of the SCNT technique remains the primary obstacle to its widespread application in buffalo [5].

Donor cells are a critical determinant of the success of animal cloning. Fibroblast cells represent the most commonly utilized donor cells for somatic cell nuclear transfer (SCNT) in animal cloning. Consequently, the establishment of fibroblast cell lines is critical for enhancing the efficiency of animal cloning [6]. The use of fibroblasts recovered from ear skin as nuclear donors provides the distinct advantage of replicating proven genetics from adult animals.

The developmental stage of the donor cell nucleus is a crucial factor influencing the development of SCNT embryos. Synchronization between the cell cycle of the donor cell nucleus and the recipient ooplasm is essential for the successful generation of a complete SCNT cloned buffalo embryo. Several studies in buffalo have demonstrated that the use of G1/G0 synchronized fibroblast cells as nuclear donors enhances the developmental competence of cloned buffalo embryos [5]. To obtain nucleated donor cells at the G0/G1 stage, donor cells are typically cultured under serum-deprived conditions. Optimization of several factors, including serum concentration, synchronization duration, and cell confluence, enhances the efficiency of donor cell nucleus synchronization.

The Thanh Chuong buffalo is an indigenous Vietnamese population. This breed demonstrates strong adaptability to the climatic and ecological conditions of Vietnam's central coastal and highland regions. Its most valuable trait is exceptional resistance to disease [7]. However, with the recent industrialization of agriculture, the demand for draught power has diminished, resulting in a decline in the population of Thanh Chuong buffalo [7]. The application of SCNT technique carries significant potential for advancing the genetic conservation of these buffalo populations in Vietnam. This study aims to optimize conditions for cell-cycle synchronization in Thanh Chuong buffalo fibroblasts, such as serum concentration, synchronization duration and cell confluence, thereby establishing a foundation for the development of cloned Thanh Chuong buffalo in Vietnam.

2. Materials and Methods

All the experimental procedures used in this study were performed following Vietnam legislation and Decision No. 2326/QĐ-BNNMT of the Ministry of Agriculture and Environment in Vietnam on June 24 2025.

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 (Currently, it is the Ministry of Agriculture and Environment) (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

Figure 1. Thanh Chuong buffalo.

Ear tissue samples were obtained from Thanh Chuong buffaloes (fifteen 1-year-old females and five 1-year-old males) in Thanh Chuong District, Nghe An Province, Vietnam (**Figure 1**). These tissue samples were rinsed with 70% ethanol to remove residual fat and hair, then transferred into Dulbecco's phosphate-buffered saline (DPBS) medium supplemented with 100 IU penicillin per mL and 100 µg streptomycin per mL. Samples were immediately transported to the laboratory. Then tissues were washed five times in DPBS, cut into 1 mm^3 fragments, and

placed at the bottom of culture plates containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cultures were maintained at 37˚C in a humidified atmosphere of 5% CO_2. After 24 hours, the medium was replaced and any detached tissue fragments were removed. Plates showing bacterial contamination were discarded. Fibroblast cultures were harvested at 80% - 90% confluence using 0.25% trypsin-EDTA and subsequently subcultured at a ratio of 1:2 or 1:3 into flasks with a growth surface area of 25 cm^2, at a seeding density of approximately 2×10^3 to 1×10^4 cells per cm^2.

2.4. Frozen Thanh Chuong Buffalo Fibroblast

Thanh Chuong buffalo fibroblasts were harvested from culture flasks at 3rd passage and counted using a hemocytometer prior to freezing. The collected cells were resuspended in a freezing medium composed of DMEM (Sigma-Aldrich, St. Louis, MO, USA), 10% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), and 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), at a final concentration of 3×10^6 to 4×10^6 viable fibroblasts per mL. Individual aliquots were dispensed into sterile 0.25-ml French straws, which were labeled with species, sex, and date, and subsequently sealed using hot forceps. The sealed straws were equilibrated at 4˚C for 20 - 30 minutes to allow DMSO penetration, then stored at −80˚C for 24 hours before being transferred to liquid nitrogen for long-term preservation.

2.5. Evaluation of the Developmental Stage of Fibroblast Nuclei

In this study, fluorescence-activated cell sorting (FACS) was employed to assess the developmental stage of nuclei in Thanh Chuong buffalo fibroblasts. Fibroblasts were treated with trypsin-EDTA, washed with phosphate-buffered saline (PBS), and resuspended in PBS to ensure complete dissociation into single cells. Fibroblasts suspensions were adjusted to a density of 1×10^6 - 1×10^7 cells per 500 µL PBS. Fibroblasts were incubated in 70% ethanol at 4˚C for 2 hours, with storage permitted at 2˚C - 8˚C or on ice. Following fixation, samples were centrifuged at 2000 rpm for 5 minutes, the ethanol was discarded, and cells were resuspended in 5 mL PBS. After 60 seconds, the suspension was centrifuged again under the same conditions, and the supernatant was removed. Fibroblasts were then resuspended in 5 mL PBS containing 0.1% Triton X-100, propidium iodide (PI) (15 2 µg per mL), and RNase A (10 µg per mL), and incubated for 15 - 20 minutes at 37˚C or 30 - 45 minutes at room temperature. Samples were analyzed using a BD FACSCanto II flow cytometer (BD Biosciences, NJ, USA) equipped with a 488-nm argon laser, with emitted fluorescence collected through filters specific for the red spectral band.

2.6. Synchronization of the Fibroblast Cycle

Fibroblasts were washed three times with DMEM medium and subsequently cultured in DMEM co-phase medium, with serum concentration, synchronization duration, and confluence adjusted according to experimental design. Fibroblasts

maintained in DMEM + 10% FBS served as the control group.

2.7. Experimental Design

All experiments in this study were conducted using Thanh Chuong buffalo fibroblasts derived from a single donor. At the point of synchronization, the cell was cultured in flasks with a 25 cm^2 growth area and concentration was approximately 4×10^4 to 8×10^4 cells per cm^2.

Experiment 1. *Evaluation of the effect of serum concentration on the cell-cycle synchronization efficiency of Thanh Chuong buffalo fibroblasts*

In *Experiment* 1, Thanh Chuong buffalo fibroblasts at 5th passages were synchronized at four serum concentrations (0.2%, 0.3%, 0.4%, and 0.5%) under conditions of 48 hours synchronization and 80% confluence. Eight biological replicates were conducted.

Experiment 2. *Evaluation of the effect of synchronization duration on the cell-cycle synchronization efficiency of Thanh Chuong buffalo fibroblasts*

In *Experiment* 2, Thanh Chuong buffalo fibroblasts at 5th passages were synchronized at five synchronization durations (24 hours, 48 hours, 72 hours, 96 hours and 120 hours) under the serum concentration conditions selected in *Experiment* 1 and with cultures maintained at 80% confluence. Eight biological replicates were conducted.

Experiment 3. *Evaluation of the effect of cell confluence on the cell-cycle synchronization efficiency of Thanh Chuong buffalo fibroblasts*

In *Experiment* 3, Thanh Chuong buffalo fibroblasts at 5th passages were synchronized at three confluence levels (60%, 80%, and 100%) under the serum concentration and synchronization duration conditions established in *Experiments* 1 and 2. Eight biological replicates were conducted.

2.8. Statistical Analysis

All data were expressed as mean ± SEM values and analysed by ANOVA, followed by Tukey's multiple comparisons test, using GraphPad Prism software (Version 7.02 for Windows, GraphPad Software, La Jolla, California, USA). $P < 0.05$ was defined as the significance level.

3. Results

3.1. Effect of Serum Concentration on the Cell-Cycle Synchronization Efficiency of Thanh Chuong Buffalo Fibroblasts

The data presented in Table 1 indicate that the proportion of nucleated fibroblasts in the G0/G1 phase of the cell cycle was highest at a serum concentration of 0.3% (81.26%) and lowest in the control group (61.44%), with the difference being statistically significant ($P < 0.05$). No significant variation was observed in the percentage of nucleated fibroblasts in the G0/G1 phase at serum concentrations of 0.2%, 0.4%, and 0.5% ($P > 0.05$; Table 1).

Table 1. Effect of serum concentration on the cell-cycle synchronization efficiency of Thanh Chuong buffalo fibroblast.

G0/G1 stage	Serum concentration				
	0.2%	0.3%	0.4%	0.5%	Control
% (Mean ± SE)	70.88^{b} ± 2.78	81.26^{a} ± 2.35	72.86^{b} ± 2.91	71.89^{b} ± 2.58	61.44^{c} ± 2.38

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

3.2. Effect of Synchronization Duration on the Cell-Cycle Synchronization Efficiency of Thanh Chuong Buffalo Fibroblasts

Based on the findings of *Experiment* 1, *Experiment* 2 employed a synchronization medium containing 0.3% serum. As shown in Table 2, the proportion of Thanh Chuong buffalo fibroblasts with nuclei in the G0/G1 phase was significantly higher in the 96-hour and 120-hour groups compared with the 24-hour, 48-hour, and 72-hour groups ($P < 0.05$). Although the percentage of fibroblasts in the G0/G1 phase of 96 hours group was higher than the 120 hours group (86.48% vs. 85.98%), however, this difference was not statistically significant ($P > 0.05$).

Table 2. Effect of synchronization duration on the cell-cycle synchronization efficiency of Thanh Chuong buffalo fibroblast.

G0/G1 stage	Culture time				
	24 hours	48 hours	72 hours	96 hours	120 hours
% (Mean ± SE)	69.46^{c} ± 2.72	81.34^{b} ± 2.66	81.98^{b} ± 2.37	86.48^{a} ± 2.65	85.98^{a} ± 2.51

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

3.3. Effect of Cell Confluence on the Cell-Cycle Synchronization Efficiency of Thanh Chuong Buffalo Fibroblasts

Based on the findings of *Experiment* 2, in this experiment, Thanh Chuong buffalo fibroblasts were synchronized using 0.3% serum for 96 hours. As presented in Table 3, the proportion of fibroblasts with nuclei in the G0/G1 phase was significantly higher in the 80% and 100% groups compared with the 60% group (86.32% and 85.91% vs 70.56%, respectively; $P < 0.05$).

Table 3. Effect of cell confluence on the synchronization efficiency of Thanh Chuong buffalo fibroblast cycle.

G0/G1 stage	Cell confluence		
	60%	80%	100%
% (Mean ± SE)	70.56^{b} ± 2.79	86.32^{a} ± 2.68	85.91^{a} ± 2.19

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

4. Discussion

The results of this study indicate that the serum concentration significantly influences the synchronization efficiency of Thanh Chuong buffalo fibroblasts. The proportion of nucleated fibroblasts in the G0/G1 phase of the cell cycle was significantly higher in the 0.3% fetal bovine serum (FBS) group compared with the 0.2%, 0.4%, and 0.5% FBS groups ($P < 0.05$). After synchronization in 0.3% FBS, 81.26% of Thanh Chuong buffalo fibroblast nuclei were arrested at the G0/G1 phase of the cell cycle (**Table 1**). These findings are consistent with those reported by Miranda *et al.* (2009) [8]. In their study, fibroblast synchronization was achieved by culturing cells in media containing serum concentrations ranging from 0.2% to 0.5%.

In this study, total cellular DNA content was quantified using a FACS system with propidium iodide (PI) staining. PI-based DNA-content flow cytometry measures overall DNA content but does not discriminate between cells in the G0 and G1 phases, as both populations possess an identical diploid DNA content (2C). Therefore, this study does not independently distinguish quiescent G0 cells from proliferative G1 cells.

Cell cycle synchronization is an essential step for the effective reprogramming of somatic cells in the somatic cell nuclear transfer (SCNT) technique. Arresting fibroblasts at the G0/G1 phase is particularly important, as this stage represents a quiescent state in which cells have exited the growth cycle and the nuclear chromatin is more accessible to reprogramming [9]. Serum starvation is the most widely used method for arresting mammalian fibroblasts in the G0/G1 phase of the cell cycle [10].

Numerous studies have demonstrated that culturing somatic cells in media supplemented with markedly reduced concentrations of fetal bovine serum (FBS), typically ranging from 0.2% to 0.5%, results in the accumulation of approximately 70% - 90% of cells in this quiescent state [11] [12]. These findings are consistent with our observations. In this study, the proportion of nucleated fibroblasts in the G0/G1 phase of the cell cycle was highest at 0.3% FBS (81.26%; $P < 0.05$; **Table 1**).

Fetal bovine serum (FBS) serves as a critical source of growth and adhesion factors, hormones, lipids, and minerals for fibroblast culture. When fibroblasts are maintained in a low-serum medium, the availability of mitogens and hormones in the extracellular environment is reduced, leading to diminished metabolic activity. Consequently, the cells revert to a resting state, with fibroblast nuclei entering the G0 phase of the cell cycle [13]. Therefore, in this study, fetal bovine serum (FBS) was employed to synchronize Thanh Chuong buffalo fibroblasts.

In the present study, synchronization duration influenced the synchronization efficiency of the cell cycle in Thanh Chuong buffalo fibroblasts. Our results demonstrate that the proportion of Thanh Chuong buffalo fibroblasts with nuclei in the G0/G1 phase increased markedly when the duration of serum deprivation was extended to 96 hours (**Table 2**). However, prolonging the synchronization period did not enhance the overall efficiency of cell-cycle synchronization in these fibro-

blasts (Table 2). Our findings are consistent with those reported by Rodrigues *et al.* (2023) [14], who observed that the proportion of cells arrested in the G0/G1 phase was highest following 96 hours of synchronization.

Although serum starvation methods have been applied across various animal species, the duration required for effective synchronization remains inconsistent, with outcomes differing according to the evaluation period and the species studied [14]. According to Młodawska *et al.* (2022) [15], the optimal synchronization period for fibroblasts is species-dependent. The proportion of cells arrested in the G0/G1 phase progressively increases with prolonged cultivation under conditions of reduced fetal bovine serum (FBS) supplementation [16]. Extended culture of fibroblasts under serum-deprived conditions diminishes the availability of growth factors, steroids, and other essential mitogenic stimuli, thereby compromising cell viability. Moreover, prolonged serum deprivation may trigger extensive DNA damage and ultimately lead to cell death [8]. This could be one of the reasons explaining the gradual decline in the proportion of nucleated fibroblasts arrested in the G0/G1 phase when serum deprivation was prolonged in this study.

In this study, cell confluence influenced the synchronization efficiency of the cell cycle in Thanh Chuong buffalo fibroblasts. In this study, the proportion of Thanh Chuong buffalo fibroblasts with nuclei in the G0/G1 phase was significantly higher in the 80% and 100% confluence groups compared with the 60% group (86.32% and 85.91% vs 70.56%, respectively, $P < 0.05$).

At present, there is no consensus among researchers regarding the optimal cell confluence required for effective fibroblast synchronization. Dalman *et al.* (2010) [17] demonstrated that full confluency is suitable for synchronizing goat fibroblasts through serum starvation. Similarly, Nguyen *et al.* (2024) [18] indicated that confluency levels exceeding 80% are suitable for the synchronization of goat fibroblasts under serum starvation. In contrast, Miranda *et al.* (2009) [8] reported that bovine fibroblasts synchronized at 60% - 70% confluency exhibited a high proportion of nuclei in the G0/G1 phase, reaching 84.1%. The variations observed between our results and those reported by other researchers may be attributable to interspecific and breed-specific characteristics, as well as differences in cell type and culture conditions employed [15].

At full confluency, the contact surface between adjacent cells progressively increases, and fibroblasts lack additional surface area for adhesion, proliferation, and growth. This condition induces contact inhibition, leading the majority of cells to exit the cell cycle and remain arrested in the G0/G1 phase [14]. Furthermore, reduced serum concentration in the synchronization medium also contributes to growth arrest, which could explain why the proportion of fibroblasts with nuclei in the G0/G1 phase does not differ significantly between synchronized at 100% confluency and those at 80% confluency in this study.

5. Conclusion

In conclusion, our findings indicate that the cell-cycle synchronization efficiency of Thanh Chuong buffalo fibroblasts was enhanced by serum deprivation at a con-

centration of 0.3%, with a synchronization period of 96 hours and confluency levels exceeding 80%.

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Author Contributions

Huong Thi Thu VU: Conceptualization, formal analysis, visualization, writing-original draft preparation; Van Khanh NGUYEN: Conceptualization, methodology, validation, writing review and editing, supervision, visualization, project administration, funding acquisition; Yen Kim Thi PHAM, Vu Thi Thu Huong: software, data curation; Au Thi HOANG, Hieu Trung Phan, Đat Van LE, Lan Anh Thi NGUYEN, Huong Le Thi NGUYEN, Duc Viet Phan: investigation, resources; Nguyen Thi Van Anh, Nguyen Thi Nhien: writing-original draft preparation; Lan Doan PHAM: supervision, writing-original draft preparation.

Conflicts of Interest

The authors declare no conflicts of interest.

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