Scientific Research Publishing

Open Journal of Animal Sciences, 2025, 15(4), 301-314
https://www.scirp.org/journal/ojas
ISSN Online: 2161-7627
ISSN Print: 2161-7597

Influence of Serum Concentration and Cell Confluency during Synchronization on the Efficiency of Cloned Ỉ Pig Embryo Production

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How to cite this paper: Hoang, A.T., Pham, Y.K.T., Vu, H.T.T., Pham, L.D., Anh, N.T.V., Ha, N.T.V., Nhien, N.T. and Nguyen, V.K. (2025) Influence of Serum Concentration and Cell Confluency during Synchronization on the Efficiency of Cloned Ỉ Pig Embryo Production. *Open Journal of Animal Sciences*, **15**, 301-314.
https://doi.org/10.4236/ojas.2025.154021

Received: July 27, 2025
Accepted: August 23, 2025
Published: August 26, 2025

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Abstract

This study aimed to assess the impact of serum concentrations and cell confluency levels during synchronization on the efficiency of Ỉ pig embryo cloning. In *Experiment* 1, the proportion of nucleated fibroblasts in the G0/G1 phase of the cell cycle was highest at serum concentrations of 0.2% and 0.3%, with no statistically significant difference between these two groups (88.75% and 85.12%, respectively; $P > 0.05$). Cleavage rates were maximized at serum concentrations of 0.1%, 0.2%, and 0.3%, yielding rates of 82.76%, 86.72%, and 85.06%, respectively. The blastocyst formation rate observed in the 0.2% group was significantly higher than that of 0%, 0.1%, 0.3%, 0.4%, 0.5%, and control groups (35.58% vs. 12.94%, 28.04%, 28.09%, 28.38%, 28.31%, and 18.41%, respectively; $P < 0.05$). Based on the results of *Experiment* 1, *Experiment* 2 utilized synchronized Ỉ pig fibroblasts cultured in 0.2% serum to examine how cell confluency influences cell cycle dynamics. The rate of fibroblasts with nuclei in the G0/G1 phase of 60% group was significantly lower than that of 80% and 100% groups (70.91% vs. 88.89% and 89.54%, respectively; $P < 0.05$). Moreover, no statistically significant differences were observed in the rate of cells in the G0/G1 phase between the 80% and 100% groups ($P > 0.05$). Among the groups studied, the 60% group demonstrated the lowest cleavage and blastocyst formation rates (69.34% and 20.94%, respectively). By contrast, no statistically significant difference in blastocyst formation was detected between the 80% and 100% groups (36.01% and 35.91%, respectively, $P > 0.05$). In conclusion, synchronizing Ỉ pig fibroblasts in 0.2% serum at 80% or 100% confluence markedly enhances the efficiency of cloned Ỉ pig blastocyst formation. The proportion of Ỉ pig fibroblasts with nuclei arrested in the G0/G1 phase

exceeded 88%, while the rate of successful cloned Ỉ pig blastocyst formation surpassed 35%.

Keywords

Ỉ Pig Fibroblast, Cycle Synchronisation, Serum Concentration, Synchronization Time, Confluence

1. Introduction

Pigs play a dual role as both economically significant livestock and vital models in biomedical research and organ transplantation, owing to their close physiological resemblance to humans (Dyck *et al.*, 2014) [1]. Despite the successful demonstration of porcine cloning via somatic cell nuclear transfer (SCNT) in 2000 (Polejaeva *et al.*, 2000) [2], its practical application in animal husbandry remains limited due to low cloning efficiency, restricted growth performance of cloned pigs, and ongoing challenges in consumer acceptance. The inefficiency observed in porcine cloning is characterized by elevated rates of miscarriage, postnatal mortality, and developmental abnormalities in cloned fetuses. These outcomes are likely attributable to incomplete nuclear reprogramming of donor cells following their transfer into enucleated oocytes (Tian *et al.*, 2003) [3], or to the failure of cloned embryos to progress beyond early developmental stages after embryo transfer (Pan *et al.*, 2015) [4].

Pre-transfer embryo quality is a critical determinant of cloning efficiency in pigs. To enhance this quality, researchers have focused on optimizing factors that influence the synchronization of donor cell cycles—particularly to increase the proportion of nuclei in the G0/G1 phase. The effectiveness of this synchronization is influenced by multiple variables, including the method employed, serum concentration, duration of synchronization, and cell confluency levels…

Fibroblasts are commonly employed as donor cells in somatic cell nuclear transfer (SCNT) embryogenesis. Serum deprivation is a widely adopted strategy for synchronizing these cells to the G0/G1 phase of the cell cycle. This approach involves reducing the serum concentration in the culture medium to a minimal level that supports cell viability while inhibiting proliferation. Serum concentration plays a pivotal role in the effectiveness of cell cycle synchronization via this method, and the optimal concentration varies according to the fibroblast subtype utilized.

Alongside serum concentration, donor cell confluency prior to synchronization plays a significant role in determining the effectiveness of cell cycle synchronization. As with serum concentration, no established consensus exists across animal species regarding optimal confluency conditions.

The Ỉ pig is a native Vietnamese breed currently listed among indigenous livestock requiring conservation due to a significant decline in population. Although

cloned Ỉ pigs have been successfully generated in Vietnam, overall cloning efficiency remains low. Previous studies have explored approaches to enhance the success of Ỉ pig embryo cloning (Van Khanh Nguyen *et al.*, 2021) [5]. However, no investigations have specifically addressed the influence of serum concentration and cell confluency on the cloning efficiency of Ỉ pig embryos. This study aims to evaluate the impact of these two factors on the efficiency of cloned embryo production in Ỉ pigs.

2. Material and Methods

All the experimental procedures used in this study were performed in accordance with Vietnam legislation and according to Decision No. 4235/QĐ-BNN-KHCN of the Ministry of Agriculture and Rural Development (currently, the Ministry of Agriculture and Environment) in Vietnam of November 11.2021.

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 Ỉ pigs at The Dabaco Pig Breeding Center, Bacninh Provine, Vietnam. Ear tissues were initially rinsed with 70% ethanol to remove residual fat and hair, then transferred into tubes containing Dulbecco's phosphate-buffered saline (DPBS) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. Samples were promptly transported to the laboratory within 2 - 3 hours. Upon arrival, tissues were washed five times in DPBS, sectioned into 1 mm^3 fragments, and plated onto culture dishes containing Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. Cultures were maintained at 37˚C in a humidified atmosphere with 5% CO_2. After 24 hours, the medium was replaced and any non-adherent tissue fragments were removed. Culture dishes exhibiting bacterial contamination were discarded imme-

diately. Once cell confluency reached 80% - 90%, cells were harvested using 0.25% Trypsin-EDTA and passaged 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)

Following synchronization, fibroblasts were treated with 0.25% Trypsin-EDTA and washed with phosphate-buffered saline (PBS). The cells were then resuspended in PBS to achieve complete dissociation, ensuring a final density of 1×10^6 to 1×10^7 cells per 500 μL PBS. Samples were fixed in 70% ethanol at 4˚C for 2 hours, with storage at 2˚C - 8˚C or on ice permitted during this period. Post-fixation, cells were centrifuged at 2000 rpm for 5 minutes, followed by ethanol removal. The pellet was resuspended in 5 ml PBS, incubated for 60 seconds, and centrifuged again under the same conditions. After discarding the supernatant, cells were permeabilized and stained in PBS containing Triton X-100, propidium iodide (PI), and RNase A, then incubated for 15 - 20 minutes at 37˚C or 30 - 45 minutes at room temperature. Fluorescence profiles were acquired using a BD FACSCanto II Flow Cytometer (BD Biosciences, NJ, USA), equipped with a 488-nm argon laser, and emission signals were captured using a red bandpass filter set.

2.5. Oocyte Collection and *in vitro* Maturation (IVM)

Oocyte collection and IVM were conducted following the protocol described by Van *et al.* (2021) [5]. Ovaries were obtained from 6 - 8-month-old prepubertal Landrace × Large White (LW) gilts at a local slaughterhouse and transported to the laboratory within 3 - 5 hours in saline solution (35˚C - 37˚C) supplemented with 0.1 mg/ml streptomycin sulfate and 100 U/ml penicillin G potassium (Sigma-Aldrich).

Upon arrival, ovaries were washed three times in Dulbecco's Phosphate-Buffered Saline (DPBS; Sigma-Aldrich) containing the same antibiotic concentrations at 37˚C. Cumulus-oocyte complexes (COCs) were retrieved by scraping visible antral follicles (≥2 mm in diameter) into TALP-HEPES medium, composed of 114 mM NaCl, 3.2 mM KCl, 2.0 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 10 mM sodium lactate, 0.1 mM sodium pyruvate, 2 mM $NaHCO_3$, 3 mg/ml bovine serum albumin, 10 mM HEPES antibiotics [100 units/ml penicillin G potassium (Sigma-Aldrich) and 0.1 mg/ml streptomycin sulfate (Sigma-Aldrich)] in 60 mm Petri dishes (Falcon 351007, Thomas Scientific, NJ, USA). COCs were collected in 60 mm Petri dishes (Falcon 351007, Thomas Scientific, NJ, USA) under a stereomicroscope. Only oocytes with intact cumulus cell layers (≥3 layers), evenly granulated cytoplasm, and no signs of lysis were selected for maturation.

Selected COCs were cultured in porcine oocyte medium (POM) [6] supplemented with 10 ng/ml epidermal growth factor (EGF, Sigma-Aldrich), 10 IU/ml eCG (Serotropin; ASKA Pharmaceutical Co., Ltd., Tokyo, Japan), and 10 IU/ml hCG (500 units; Puberogen, Novartis Animal Health, Tokyo, Japan) throughout the entire IVM to the report of Van *et al.* (2021) [5]. To synchronize maturation,

1 mM dibutyryl cAMP (dbcAMP; Sigma) was added during the first 22 h of IVM. Cultures were maintained in 4-well dishes (Nunc MultiDishes, Thomas Scientific) with 500 µl IVM medium per well, overlaid with mineral oil (Sigma-Aldrich), and incubated at 39˚C in a gas mixture of 5% CO_2, 5% O_2, and 90% N_2. After 22 h, COCs were transferred to dbcAMP-free maturation medium and cultured for an additional 22 - 24 h under the same conditions. Each well contained 30 - 50 COCs.

2.6. SCNT and Embryo Culture

SCNT embryos were generated using a modified zona-free method as previously described [5]. Following 40 - 42 hours of in vitro maturation, cumulus cells were removed from oocytes by vortexing in TALP-HEPES medium containing 1 mg/ml hyaluronidase (Sigma-Aldrich). The TALP-HEPES medium consisted of 114 mM NaCl, 3.2 mM KCl, 2.0 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 10 mM sodium lactate, 0.1 mM sodium pyruvate, 2 mM $NaHCO_3$, 3 mg/ml bovine serum albumin and 10 mM HEPES.

Matured oocytes exhibiting the first polar body were selected and treated with 0.5% Pronase (Sigma-Aldrich) in TALP-HEPES for 3 - 6 minutes at 38.5˚C to remove the zona pellucida. Zona-free oocytes were washed in TALP-HEPES supplemented with fetal bovine serum (FBS) and maintained in PZM3 medium [7] containing 2.5% FBS until enucleation.

To induce chromosome plate protrusion, zona-free oocytes were incubated in PZM3 medium supplemented with 4 µM demecolcine (Sigma-Aldrich) and 2.5% FBS for 20 - 40 minutes. Enucleation was performed in TALP-HEPES medium containing 7.5 µg/ml cytochalasin B (Sigma-Aldrich) [8], using a blunt pipette and a closed-holding pipette to remove metaphase chromosomes.

Post-enucleation, cytoplasts were briefly exposed to 300 µg/ml phytohemagglutinin (Sigma-Aldrich) in HEPES-buffered TCM-199 [9] and immediately placed over a single donor cell in a 100 µl drop of TALP-HEPES medium. The cytoplast-donor cell couplets were then transferred to fusion medium for 2 - 3 minutes before being placed in a fusion chamber containing 2 ml of pre-warmed fusion medium composed of 0.3 mM mannitol, 0.05 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MgSO_4 \cdot 7H_2O$ and 25 mg/ml PVA.

Fusion was achieved using a double DC pulse of 70 V for 30 µsec with an electro-cell fusion system (LF 101, Nepa Gene Co., Ltd., Chiba, Japan). Fused couplets were cultured in PZM3 medium supplemented with 2.5% FBS at 38.5˚C under 5% CO_2 and 5% O_2 in humidified air. Fusion was confirmed 30 - 50 minutes post-treatment; non-fused couplets were discarded. Fused embryos were incubated for 2 hours to allow reprogramming, followed by activation via a single DC pulse of 65 V for 80 µsec. Subsequently, embryos were cultured for 3 hours in PZM3 medium containing 7.5 µg/ml cytochalasin B under the same incubation conditions.

SCNT embryos were cultured using a modified Well-of-the-Well (WOW) system [10]. Microwells were created in 35 mm Petri dishes (Corning, USA) by indenting the surface with a heated steel needle. Each of the 20 microwells was cov-

ered with a 100 µl drop of PZM3 medium overlaid with mineral oil, and a single SCNT embryo was placed in each well. Culture was maintained at 38.5˚C in 5% CO_2 and 5% O_2 in humidified air. The day of activation was designated as Day 0. Cleavage was assessed 48 hours post-activation, and the development of compacted morulae and blastocysts was recorded on Days 5, 6, and 7. On Day 5, the culture medium was supplemented with 10% (v/v) FBS.

2.7. Synchronization of the Fibroblast Cycle

Fibroblasts were washed three times with Dulbecco's Modified Eagle Medium (DMEM) and subsequently cultured in DMEM co-phase medium under experimental conditions that varied in serum concentration and cell confluency. Fibroblasts cultured in DMEM supplemented with 10% serum served as the control group.

2.8. Evaluation of Total Cell Number in Blastocyst Stage Embryos

On Day 7 post-activation, the total cell number of blastocysts was determined using Hoechst 33342 staining. Blastocysts were first washed in phosphate-buffered saline (PBS) supplemented with 0.3% polyvinylpyrrolidone (PVP) to remove residual culture medium. Subsequently, embryos were incubated overnight at 4˚C in a staining solution consisting of Hoechst 33342 and absolute ethanol at a 1:9 ratio. Following staining, embryos were rinsed in absolute ethanol and transferred into a glycerol solution for mounting. Each embryo was individually placed in a droplet on a glass slide, oriented longitudinally, and covered with a coverslip. Stained embryos were then examined under a fluorescence microscope, and total cell numbers were recorded based on nuclear fluorescence.

2.9. Experiment Design

Experiment 1: *The effect of serum concentration on cell cycle of Ỉ pig fibroblasts and the subsequent development of SCNT embryos*.

This experiment was performed to assess the effect of serum concentration on the nuclear status of Ỉ pig fibroblasts and the subsequent development of SCNT embryos. In this experiment, Ỉ pig fibroblasts were synchronized at six different serum concentrations 0%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% under conditions of 80% confluency and a 48-hour synchronization period. In the control group, Ỉ pig fibroblasts were synchronized using 10% fetal calf serum (FCS) under the previously described conditions. Nuclear status of cells was compared between the groups as described above. SCNT embryos were cultured in PZM3 medium [8] for 7 days. On Day 5 (Day 0 = SCNT), 10% (v/v) fetal bovine serum was added to culture drops. Cleavage rates recorded on Day 2 (Day 0 = SCNT) and blastocyst (**Figure 1**) rates recorded on Day 7 were compared among 7 groups. Ten replications were performed.

Experiment 2: *The effect of cell confluency levels on cell cycle of Ỉ pig fibroblasts and the subsequent development of SCNT embryos*.

This experiment was performed to assess the effect of cell confluency levels on the nuclear status of Ỉ pig fibroblasts and the subsequent development of SCNT embryos. In this experiment, Ỉ pig fibroblasts were synchronized at three distinct cell confluency levels: 60%, 80%, and 100% (**Figure 2**) under conditions of a 48-hour synchronization period and using the serum concentration determined from the results of *Experiment* 1. Nuclear status of cells, cleavage and blastocyst formation rates were compared among the groups as described in *Experiment* 1.

2.10. Data Analysis

All data were expressed as mean ± SEM values and analysed by ANOVA, followed by Tukey's multiple comaprisons 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 Cell Cycle of Ỉ Pig Fibroblasts

As presented in **Table 1**, the proportion of nucleated fibroblasts in the G0/G1 phase of the cell cycle was highest at serum concentrations of 0.2% and 0.3%, with no statistically significant difference between these two groups (88.75% and 85.12%, respectively; $P > 0.05$). The highest proportion of nucleated fibroblasts in the G0/G1 phase of the cell cycle was observed at a serum concentration of 0.2% (88.75%), while the lowest was recorded at 0% serum (24.69%), with the difference being statistically significant ($P < 0.05$).

3.2. Effect of Serum Concentration on the Subsequent Development of SCNT Embryos

The data presented in **Table 2** indicate that serum concentration significantly influences the subsequent development of SCNT-derived Ỉ pig embryos. As shown in **Table 2**, the cleavage rates were highest in the 0.1%, 0.2%, and 0.3% serum concentration groups, with values of 82.76%, 86.72%, and 85.06%, respectively. However, the 0.2% group exhibited a significantly higher blastocyst formation rate compared to the 0%, 0.1%, 0.3%, 0.4%, 0.5%, and control groups (35.58% vs. 12.94%, 28.04%, 28.09%. 28.38%, 28.31% and 18.41%, respectively, $P < 0.05$).

3.3. Effect of Cell Confluency Levels on Cell Cycle of Ỉ Pig Fibroblasts

Based on the findings from Experiment 1, this experiment synchronized Ỉ pig fibroblasts using 0.2% serum to evaluate the effect of varying cell confluency levels on the cell cycle dynamics of Ỉ pig fibroblasts. As demonstrated in **Table 3**, the pre-synchronization cell confluency levels of Ỉ pig fibroblasts significantly influence synchronization efficiency. Specifically, the proportion of Ỉ pig fibroblasts with nuclei in the G0/G1 phase was markedly lower in the 60% confluency group compared to the 80% and 100% confluency groups (70.91% vs. 88.89% and

89.54%, respectively; $P < 0.05$). Although the proportion of Ỉ pig fibroblasts with nuclei in the G0/G1 phase was higher in the 100% confluency group compared to the 80% confluency group, however, this difference was not statistically significant ($P > 0.05$).

3.4. Effect of Cell Confluency Levels on the Subsequent Development of SCNT Embryos

The results of **Table 4** demonstrates that cell confluency has a significant impact on the developmental outcomes of SCNT-derived Ỉ pig embryos. Embryos derived from the 60% confluency group exhibited the lowest cleavage and blastocyst formation rates (69.34% and 20.94%, respectively). In contrast, no statistically significant difference was observed in the blastocyst formation rates between the 80% and 100% confluency groups (36.01% and 35.91%, respectively, $P > 0.05$).

4. Discussion

The efficiency of somatic cell nuclear transfer (SCNT) is influenced by multifaceted interactions among various factors, with cell cycle synchronization between the donor nucleus and the recipient oocyte cytoplasm serving as a critical determinant of its success. Effective synchronization between the cell cycle of donor nuclei and the cytoplasmic of recipient oocytes is widely acknowledged as a critical determinant for preserving genomic integrity and appropriate ploidy in somatic cell nuclear transfer (SCNT) embryos. Donor cells in a quiescent state, specifically arrested in the G0/G1 phases of the cell cycle, are frequently employed in the generation of cloned animals [11]. Several studies have suggested that the utilization of donor cells arrested in the G0/G1 phase is essential for complete nuclear reprogramming, contributing to the development of high-quality embryos [12].

Serum starvation and growth arrest upon reaching confluence are additional techniques commonly employed to synchronize cultured cells at the G0/G1 phase of the cell cycle. Serum starvation is a widely utilized method for inducing cell cycle synchronization in preparation for cloning procedures, and is commonly applied to arrest mammalian fibroblasts at the G0/G1 phase [13] [14]. Cultured fibroblasts typically require sufficient nutritional support to progress through mitosis, with serum commonly employed in animal cell culture systems. Serum provides essential growth and adhesion factors, hormones, lipids, and minerals necessary for maintaining cellular functions *in vitro* [15]. Serum starvation for several days, which reduces proliferative stimuli such as growth factors, is an effective method for inducing cellular entry into the G0/G1 phase of the cell cycle [13]. Under low-serum conditions, the availability of mitogens and hormones diminishes, leading to reduced cellular metabolism and the induction of a quiescent state (G0 phase) [16]. Serum deprivation during cell culture eliminates mitotic signaling, prompting a rapid exit from the cell cycle and entry into the quiescent G0 phase, characterized by diminished metabolic activity. Lowering serum levels in the culture medium also accelerates cellular protein degradation, further facil-

itating the transition of cells into the G0 state [17]. Studies across multiple species have demonstrated that, when metaphase II enucleated oocytes are employed as recipient cytoplasts in SCNT, the donor somatic cell nucleus must be in the G0/G1 phase of the cell cycle to support optimal embryonic development [18].

Our results demonstrate that synchronizing Ỉ pig fibroblasts using 0.2% serum yields the highest proportion of cells with nuclei arrested in the G0/G1 phase of the cell cycle, relative to those cultured at serum concentrations of 0%, 0.1%, 0.3%, 0.4%, and 0.5% (**Table 1**).

Table 1. Effect of serum concentration on cell cycle of Ỉ pig fibroblasts.

Concentration	% of cells at different stages of the cell cycle			
	G0/G1	G2/M	S	Non defined
0%	24.69^{a} ± 2.01	5.08 ± 2.68	1.37 ± 1.12	68.86 ± 3.42
0.1%	67.89^{c} ± 1.92	8.56 ± 2.61	3.91 ± 2.08	19.64 ± 1.98
0.2%	88.75^{e} ± 1.64	6.32 ± 1.99	2.24 ± 1.81	2.69 ± 2.12
0.3%	85.12^{e} ± 1.75	6.21 ± 1.72	3.01 ± 1.89	5.66 ± 2.05
0.4%	78.26^{d} ± 2.21	12.34 ± 2.04	3.98 ± 2.65	5.42 ± 1.83
0.5%	74.18^{d} ± 1.85	14.54 ± 1.96	6.48 ± 1.72	4.8 ± 2.08
Control	69.36^{b} ± 1.89	11.14 ± 2.76	12.82 ± 2.34	6.68 ± 1.34

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

In somatic cell nuclear transfer (SCNT) for cloning, using donor cells in the G0/G1 phase of the cell cycle is generally preferred. When the nucleus of the donor cell is not in the G0 or G1 phase, it can lead to chromosomal abnormalities (aneuploidy), which are detrimental to embryonic development [19]. Addition, the cells in the G0/G1 phase are less likely to have issues with chromosome pulverization or aneuploidy during reprogramming, which can occur in other phases. This observation accounts for the significantly higher blastocyst formation rate observed in the 0.2% group relative to the other experimental concentrations (**Table 2**).

Consequently, in subsequent experiments aimed at generating SCNT embryos, Ỉ fibroblasts cultured in serum-deprived medium (0.2%) at were utilized as donor cells.

In *Experiment 2*, we evaluated the effects of cell confluency on the synchronization of Ỉ pig fibroblasts and examined the subsequent *in vitro* developmental competence of SCNT embryos. The analysis revealed no statistically significant differences in the G0/G1 phase of the cell cycle (88.89% vs. 89.54%, respectively, $P > 0.05$, **Table 3**) or in blastocyst formation between the 80% and 100% groups (36.01% vs. 35.91%, respectively, $P > 0.05$, **Table 4**).

Table 2. Effect of serum concentration on the subsequent development of SCNT embryos.

Concentration	Total	Cleaved (% total)	Blastocyst (% total)	The average total cells per blastocyst
0	320	191 $59.92^{a} \pm 2.34$	40 $12.94^{a} \pm 2.12$	49.62 ± 1.89
0.1	341	280 $82.76^{c} \pm 2.08$	95 $28.04^{c} \pm 2.46$	49.67 ± 2.36
0.2	336	289 $86.72^{c} \pm 2.19$	118 $35.58^{d} \pm 2.26$	50.69 ± 2.45
0.3	328	278 $85.06^{c} \pm 2.41$	91 $28.09^{c} \pm 2.56$	50.38 ± 2.48
0.4	330	230 $70.08^{b} \pm 1.96$	92 $28.38^{c} \pm 2.43$	49.97 ± 2.16
0.5	346	242 $70.21^{b} \pm 2.51$	97 $28.31^{c} \pm 2.26$	50.01 ± 2.31
Control	338	177 $51.86^{a} \pm 2.25$	61 $18.41^{b} \pm 2.76$	48.98 ± 2.29

Ten 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 cell confluency levels on cell cycle of Î pig fibroblasts.

Confluency levels	G0/G1	G2/M	S	Non defined
60%	$70.91^{a} \pm 2.46$	4.65 ± 2.14	2.42 ± 2.09	18.02 ± 2.69
80%	$88.89^{b} \pm 2.15$	5.27 ± 2.82	2.85 ± 1.76	2.99 ± 2.44
100%	$89.54^{b} \pm 2.64$	5.81 ± 2.38	2.04 ± 2.56	2.61 ± 1.96

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

Table 4. Effect of cell confluency levels on the subsequent development of SCNT embryos.

Confluency levels	Total	Cleaved (% total)	Blastocyst (% total)	The average total cells per blastocyst
60%	426	295 $69.34^{a} \pm 2.09$	89 $20.94^{a} \pm 2.75$	50.82 ± 2.21
80%	418	280 $87.02^{b} \pm 2.41$	150 $36.01^{b} \pm 2.61$	51.07 ± 2.74
100%	420	289 $86.51^{b} \pm 2.26$	150 $35.91^{b} \pm 2.36$	50.96 ± 2.31

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

These findings are consistent with previous studies conducted by Van *et al.* (2024) [20], Sun *et al.* (2008) [21], and Dalman *et al.* (2010) [22]. Van *et al.* (2024) [20] reported that synchronization of goat fibroblasts at complete confluency (100%) led to 87.17% of nucleated cells being arrested in the G0/G1 phase of the cell cycle. Sun *et al.* (2008) [21] reported that synchronizing bovine fibroblasts at full confluency (100%) resulted in 91.5% of nucleated cells being arrested in the G0/G1 phase. Similarly, Dalman *et al.* (2010) [22] demonstrated that complete confluency, combined with serum starvation, is an effective strategy for inducing cell cycle synchronization in goat fibroblasts.

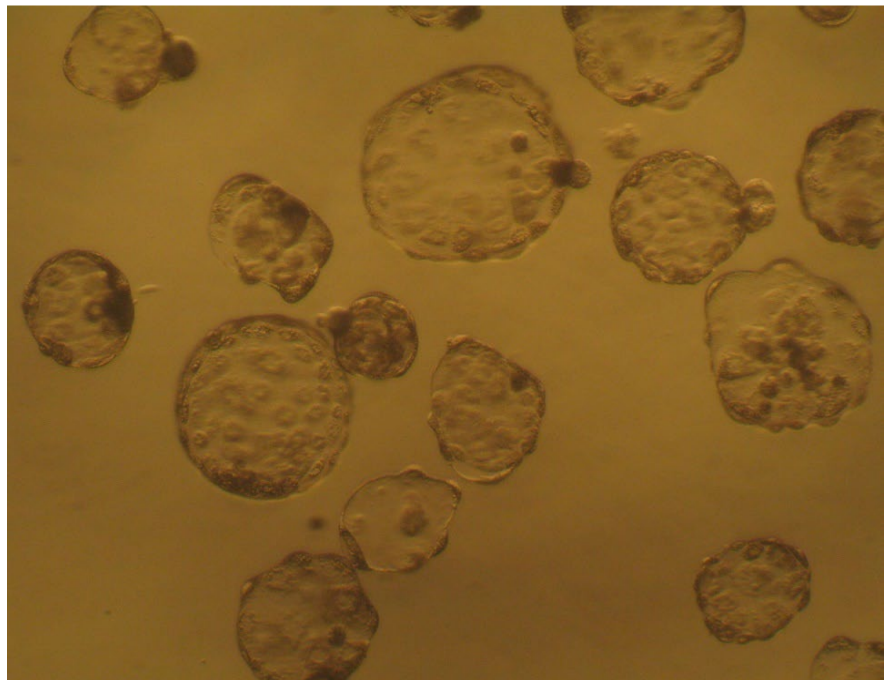

Figure 1. Cloned Ỉ pig blastocysts at Day 7 after SCNT (eyepiece * objective is 10×/0.25).

The degree of cell confluency can be utilized as a strategy to synchronize fibroblasts, facilitating the alignment of cells at various stages of the cell cycle into a uniform phase. Specifically, attaining high confluency levels (typically exceeding 80%) effectively arrests fibroblasts in the G0/G1 phase, rendering them suitable donor cells for somatic cell nuclear transfer (SCNT). This approach is frequently preferred over other synchronization techniques, such as serum starvation, due to its lower induction of apoptosis. At full (100%) confluency, fibroblasts lack available surface area for further adhesion, proliferation, and development. Consequently, a proportion of the cell population enters a temporary quiescent state, characterized by the cessation of cell division and reduced proliferative activity. In addition, some fibroblasts may cease proliferation due to the low serum concentration in the culture medium, which could contribute to the observed higher proportion of nucleated fibroblasts in the G0/G1 phase within the 100% confluency group compared to the 80% and 60% confluency groups [20].

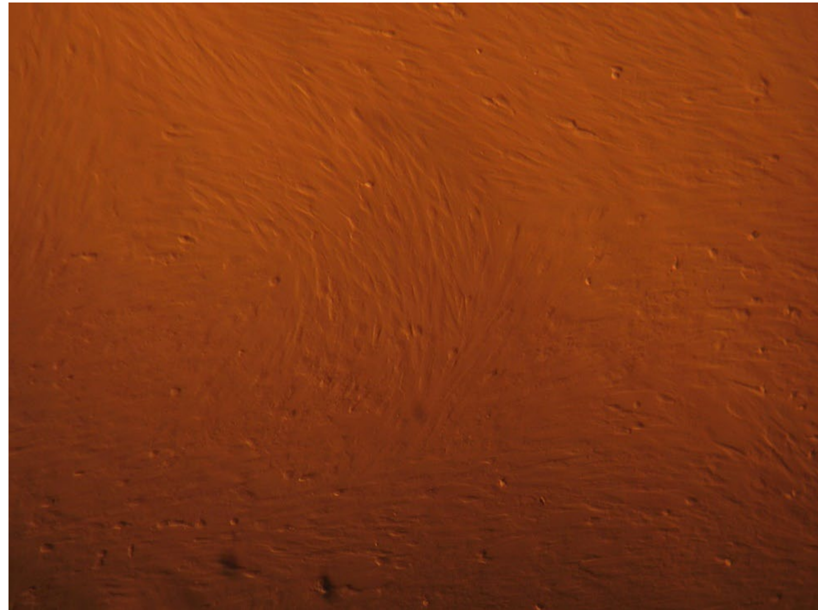

Figure 2. Morphology of Ỉ pig fibroblasts cultured at 100% confluency (eyepiece * objective is 10x/0.25). Cells exhibit a densely packed, monolayer arrangement across the culture surface, indicating full confluency and limited available space for further proliferation.

These findings indicate that Ỉ pig fibroblasts were effectively synchronized using the serum deprivation method, employing a reduced serum concentration of 0.2% at either 80% or 100% confluency. Under these conditions, the rate of blastocyst formation exceeded 80%. As reported by Miranda *et al.* (2009) [13], achieving a G0/G1 nuclear arrest in more than 80% of fibroblasts is considered sufficient for nuclear transfer and the generation of cloned embryos.

5. Conclusion

These findings indicate that synchronization of Ỉ pig fibroblasts in 0.2% serum under conditions of 80% or 100% confluence significantly improves the efficiency of cloned blastocyst production. The proportion of Ỉ pig fibroblasts with nuclei arrested in the G0/G1 phase exceeded 88%, while the rate of successful cloned Ỉ pig blastocyst formation surpassed 35%.

Acknowledgements

The work was carried out at the Key Laboratory of Animal Cell Technology-National Institute of Animal Sciences with financial support from the Vietnamese Ministry of Agriculture and Environment-Vietnam.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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