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REVIEW ARTICLE

IMPROVING THE EFFICIENCY OF BOVINE SOMATIC CELL NUCLEAR TRANSFER PROTOCOL BY ESTABLISHMENT OF CULTURE MEDIUM CHANGING SYSTEM

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ABSTRACT

Bovine cloning is expected as a promising technology for endangered genome conservation and the production of transgenic cloned cows to manufacture human recombinant proteins. However, cloned bovine embryos often resulted in poor development, thus reducing the success of producing cloned offspring. One of the reasons is based on culture medium, which directly relates to the development of cloned bovine embryos. This study aimed to establish culture medium changing system to improve the efficiency of bovine somatic cell nuclear transfer protocol. Our results showed that using the culture medium of Takahashi and First 1992 produced the cloned bovine blastocyst at the highest rate (9.4%, $P < 0.05$) among different modified synthetic oviduct fluid (mSOF) media. In addition, the rate of embryos developed to the hatching blastocyst was highest in the culture group within transfer embryos to the fresh medium with 5% FBS on day 3 and day 5 (18.5%) compared to the other treatments. Finally, we found that cloned embryos in the group of partial replacement presented significantly higher blastocyst formation rate and quality compared to the group of full replacement (27.6% and 15.6%, respectively, $P < 0.05$), and it also showed the highest number of cells in blastocyst (about 116 cells). In conclusion, using mSOF and changing the fresh medium supplemented with 5% FBS on day 3 following partial replacement on day 5 improved the preimplantation development of cloned bovine embryos and promoted the blastocyst quality.

KEYWORDS

cloned bovine embryos, full replacement, mSOF culture medium, partial replacement, preimplantation development.

1. INTRODUCTION

Since the success of Dolly the sheep, somatic cell nuclear transfer (SCNT) has become a powerful technique for extensive application in industrial farm animal production, bio-organ transplantation, conservation of valuable genetic resources, and manufacturing human recombinant protein (Wilmut et al., 1996; Knosalla et al., 2018; Borges et al., 2019; Wu et al., 2012; X Su et al., 2018). Unfortunately, the success rate to birth was very low (~2-3%) in bovine cloning, leading to a severe impediment in the practical use of the SCNT technique (Rodriguez-Osorio et al., 2012). Among the strategies for improving the cloning efficiency, optimizing the vitro culture system is a critical factor to determine the efficiency of embryo developmental competence. Several culture media have been used for culturing of bovine embryos to develop from zygote to blastocyst, including TCM199, CR1aa and CR2, and IVD101, etc. (Gandhi., 2000; Rosenkrans and First et al., 1994; Abe and Hoshi., 2003).

Until now, the most widely accepted medium was modified synthetic oviduct fluid (mSOF) because of its simple composition and ability to support the development of bovine embryos both in vitro and in vivo (Holm et al., 1999). However, the formulation of SOF medium differed in many studies (Tervit et al., 1972; Holm et al., 1999; Takahashi and First, 1992). These formulations varied in osmolarity, salt compositions, energy substrate concentration, and presence of myo-Inositol and sodium citrate in mSOF of Takahashi and First. Still, there is no comparison between the effect of different mSOF media on development of cloned bovine embryos.

It is well known that supplementation with fetal bovine serum (FBS) to the culture medium is beneficial for bovine embryo development (Carolan et al., 1995). In addition, 5% FBS has been evidently demonstrated for its significant effects served as growth factors and energy sources to promote post-cleavage embryo development in bovines (Wang et al., 1997). Although previous research has demonstrated the essential use of FBS supplementation, bovine embryo arrest often occurs at the 8-cell stage (Zhao et al., 2009).

Therefore, its effect on different stages during preimplantation development of cloned bovine embryos was not clearly examined. Based on these issues, the first aim of this study is to examine and compare the effects of different mSOF media on the preimplantation development of cloned bovine embryos and blastocyst quality. Secondly, we aimed to examine the effect of the defined mSOF medium supplemented with 5% FBS at different duration timing from pronuclear formation stage to blastocyst stage based on developmental competence of cloned bovine embryos and blastocyst quality. Furthermore, after defining the optimal culture system for cloned bovine embryos, we evaluated the effect of the medium changing methods either by full or partial replacement of fresh medium supplemented with 5% FBS to the culture system on blastocyst formation rates. In summary, this study established the in-vitro bovine embryo culture system to maximize the cloned blastocyst production at high quantity and quality for further processes to produce cloned cows with high efficiency.

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2. Material and Method

2.1 Sample Preparation and Somatic Cell Nuclear Transfer

In-vitro maturation (IVM) of bovine oocyte and donor cell preparation were performed as previous describe (Thuan et al., 2019). In brief, the cumulus-oocyte complexes (COCs) were cultured in IVM medium covered with mineral oil in a humidified 5% CO_2 incubator at 38.5°C. After 18 – 20h of culture, COCs were expanded, and surrounding cumulus cells were removed from oocytes by treatment with hyaluronidase and mouth pipetting. The oocytes extruded the first polar body (1st PB) with homogenous cytoplasm were considered matured and used for SCNT. Donor bovine lung tissues were washed several times in absolute ethanol and phosphate-buffered saline (PBS) to avoid contamination. Afterward, tissues were dissected into small pieces and were seeded in a cell culture disk of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS, 100 IU/mL Penicillin in a humidified 5% CO_2 incubator at 38.5°C. After reaching 80% confluence, cells were passed into the next passage and were frozen. The cell culture at the passage 3-7 were thawed and treated with trypsin before nuclear transfer.

SCNT was performed based on the established protocol from Cellular Reprogramming Laboratory with some modification (Thuan et al., 2019). In brief, matured oocytes were enucleated by aspirating the metaphase II chromosome in HEPES medium supplemented with 7.5 µg/mL cytochalasin B under a micromanipulator system using an XY clone laser to open the zona pellucida of oocytes. After that, the prepared fibroblast cells were transferred to the enucleated oocytes by microinjection using the piezo action. After recovery within 2 hours in mSOF, the reconstructed oocytes were activated to become cloned embryos by 5 µM Ionomycin for 5 mins at room temperature (RT), followed by treatment of 2 mM 6-(Dimethylamino)purine (6-DMAP) in mSOF medium for 4 hours at 38.5°C, 5% CO_2 and 95% humidified air. Embryos then were in vitro developed (IVD) within the two-steps treatment of HDACi (purpose for supporting high developmental competence of cloned bovine embryos) (Thuan NV et al., unpublished data) in droplets (each drop contains 10 µL) of different mSOF media. The different mSOF media were prepared following the compositions in the Table 1 without non-essential, essential amino acid, bovine serum albumin and were stored at 4°C for two months. The culture media supplemented with non-essential, essential amino acid, bovine serum albumin were stored at 4°C and used within one week.

Table 1: The Composition of Different mSOF Media

SOF Composition	Modified from Takahashi & First 1992		Modified from Holm et al., 1999		Modified from Tervit et al., 1972	
NaCl	95	mM	107.63	mM	107.7	mM
KCl	7.16	mM	7.16	mM	7.16	mM
KH_2PO_4	1.19	mM	1.19	mM	1.19	mM
$MgCl_2.6H_20$	0.49	mM	-		0.49	mM
$MgSO_4.7H_2O$	-		1.51	mM	-	
Sodium pyruvate	0.3	mM	5.35	mM	0.33	mM
L-Glutamine	1	mM	7.27	mM	-	
Sodium lactate	3.3	mM	0.2	mM	3.3	mM
Glucose	-		-		1.5	mM
Tri-sodium citrate	0.34	mM	0.34	mM	-	
Myo-Inositol	2.77	mM	2.77	mM	-	
Kanamycin	50	µg/ml	50	µg/ml	50	µg/ml
$NaHCO_3$	25.07	mM	25	mM	25.07	mM
$CaCl_2.2H_20$	1.71	mM	1.78	mM	1.71	mM
100X Non-Essential Amino Acids	1	%	1	%	1	%
50X essential Amino Acids	2	%	2	%	2	%
Bovine serum albumin	0.3	%	0.3	%	0.3	%

2.2 Supplementation of 5% FBS to mSOF Medium for Cloned Bovine Embryos Culture

After defining the ideal mSOF medium for cloned bovine embryos culture, 5% FBS was supplemented to the defined mSOF medium at different duration timing (culturing embryos were transferred to the fresh mSOF supplemented with 5% FBS one time on day 3 or two times on day 3 and day 5 after activation) and the developmental rates from pronuclear formation to the blastocyst stage (7 days) were recorded and compared among groups.

2.3 Partial and Full Replacement (Changing Medium Methods) of Fresh mSOF Supplemented with 5% FBS to Cloned Bovine Embryos Culture Medium at Day 5 Post-Activation (PA)

The medium changing methods at day 5 PA of embryos culture were examined between partial replacement (use mouth pipette to take out 50% of medium and add 50% fresh medium supplemented with 5% FBS into the culture drops) and full replacement (use mouth pipette to transfer the cloned embryos to a new droplet of fresh medium supplemented with 5% FBS).

2.4 Evaluation Quality of Cloned Blastocyst

On day 7 PA, cloned bovine blastocyst were fixed with 4% paraformaldehyde for 30 minutes at RT. After fixation, embryos were washed in PBS-BSA twice (10 minutes each time). For allowing permeabilization of staining dye into the cell membrane, embryos were treated with 0.5% Triton X-100 in PBS-BSA for 1 hour. Then the samples were washed in PBS-BSA twice (10 minutes each time). After that, each group was DNA staining with 2 µg/mL of 4', 6-diamidino-2phenylindole dihydrochloride (DAPI, blue) for 30 minutes. Finally, samples were washed twice with PBS- BSA (10 minutes each time) before being mounted on microscopic slides using glycerol covered with a glass coverslip. Each slide was observed under Nikon Eclipse Ti-U to count the number of cells in each blastocyst by NIS- Element BR Analysis 4.500.000 64-bit Program.

2.5 Statistical Analysis

Each experiment was repeated at least three times. All experiment data were analyzed by one-way ANOVA (analysis of variance) using the SPSS version 20. A probability of $P < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 Effect of Different mSOF Culture Media on Preimplantation Development of Cloned Bovine Embryos

According to Table 2, cloned bovine embryos cultured in mSOF from Takahashi & First resulted in the highest developmental rate to the blastocyst (9.4%) compared to the other mSOF groups (2.7% and 8.3%). This result might be due to the presence of sodium citrate and myo-Inositol. The earlier report has proven that sodium citrate has an embryotropic effect. Citrate facilitates fatty acid synthesis and plays a role as a chelator of metal ions (e.g., Ca^{2+}) (Keskintepe et al., 1995; Goodridge

1973). This effect might be critical for maintaining junctional integrity. Consequently, it is essential for compaction and blastocyst formation (Gray et al., 1992). Additionally, adding tri-sodium citrate to mSOF also improved blastocyst development (Holm et al., 1999).

Table 2: Effect of Different mSOF Culture Medium on Preimplantation Development of Cloned Bovine Embryos

Culture Medium	No. of Embryos Examined	No. (%) of Cloned Bovine Embryos Developed to			
		2-cell	4-cell	8-cell	Blastocyst
Modified SOF from Tervit et al 1972	37	18 (48.6)[a]	12 (32.4)[a]	7 (18.9)[a]	1 (2.7)[a]
Modified SOF from Holm et al 1999	24	18 (75.0)[b]	16 (66.7)[b]	12 (50.0)[b]	2 (8.3)[ab]
Modified SOF from Takahashi & First 1992	32	27 (84.4)[b]	26 (81.2)[b]	22 (68.8)[b]	3 (9.4)[b]

Within the same column, percentages with different superscripts (a, b, and c) differ significantly (P < 0.05). Three replicates were performed to obtain the data.

The presence of glucose in Tervit formulation could be harmful to the embryos (Takahashi and First, 1992). Though it has been unclear how glucose affects cattle and various species, glucose is supposed to prevent respirators with phosphate and cause the ineffective production of ATP during embryo development (Schini et al., 1988). Besides, the concentration of NaCl in mSOF was also a critical aspect. The NaCl concentration was supposed not to be over 95 mM and maintains the total osmolarity of the medium from 250 to 270 mOsm, whereas both Holm and Tervit formulations were above this level (Liu et al., 1996). NaCl with high concentrations prefers to hyperosmolarity primarily and depressed embryo development. Thus, the preimplantation development of cloned bovine embryos might be affected due to these factors, and mSOF from Takahashi and First was considered the most suitable medium for cloned bovine embryo culture.

3.2 Effect of mSOF Medium Supplemented with 5% FBS at Different Duration Timing on Preimplantation Development of Cloned Bovine Embryos

Table 3: Effect of mSOF Medium Supplemented with 5% FBS at Different Duration Timing on Preimplantation Development of Cloned Bovine Embryos

Treatment	No. of Embryos Examined	No. (%) of Cloned Bovine Embryos Developed to			
		Morula	Early Blastocyst	Hatching Blastocyst	Cells Number
Keep in mSOF for 7 days	31	5 (16.1)[a]	3 (9.6)[a]	0 (0.0)[a]	0 (0.0)[a]
Change fresh medium on day 3	25	5 (20.0)[ab]	2 (8.0)[a]	0 (0.0)[a]	0 (0.0)[a]
Change fresh medium with 5% FBS on day 3	24	6 (25.0)[ab]	4 (16.7)[ab]	2 (8.3)[b]	72 (±5.3)[b]
Change fresh medium with 5% FBS on day 3 and day 5	27	8 (29.6)[b]	6 (22.2)[b]	5 (18.5)[c]	95 (±6.8)[c]

Within the same column, percentages with different superscripts (a, b, and c) differ significantly (P < 0.05). Three replicates were performed to obtain the data.

The data from Table 3 shows that transferring cloned embryos to a droplet of fresh medium supplemented with 5% FBS on day 3 and day 5 PA produced the number of morulae that was significantly higher than the control group without any changing medium and 5% FBS supplementation (29.6% compare to 16.1%, respectively). In addition, the rate of cloned bovine embryos developed to the blastocyst stage was highest in a culture group with changing the fresh medium with 5% FBS on both day 3 and day 5 PA (18.5% expanded blastocyst) compared to the other treatments without supplementation of 5% FBS (0%) and changing the fresh medium with 5% FBS only on day 3 (8.3%). In other words, when changing the fresh medium with 5% FBS on day 3 and day 5 PA, the number of cloned bovine embryos developed to blastocyst was increased.

It is well known that FBS contains vitamins, hormones, and growth factors which are essential for the preimplantation development of bovine embryos (Wang et al., 1997). Besides, it is beneficial for embryo development in cattle when adding amino acids to the culture medium (mSOF) (Takahashi and First, 1992). However, spontaneously breaking down at 37-39°C of amino acid and the metabolism of amino acid by embryos resulted in the medium a high level of ammonium, and the toxicity of ammonium on cells has been well reported. Additionally, it also inhibited embryo development (Gardner et al., 1993). In our study, ammonium and other toxic factors in the culture medium could be removed by transferring cloned embryos to a fresh medium (changing medium).

3.3 Effect of Changing Medium Methods on Preimplantation Development of Cloned Bovine Embryos

Within the same column, percentages with different superscripts (a, b, and c) differ significantly (P < 0.05). Three replicates were performed to obtain the data.

In the developmental rates comparison of the cloned bovine embryo during preimplantation development between two changing medium methods, there were significant differences between 2 groups at both morula and blastocyst stage (Table 4). After changing medium at day 5 PA, only 28.1% of cloned embryos developed to morula, and the rate decreased to 15.6% blastocyst formation in the full replacement group. On the other hand, the percentage of morula formation peaked at more than 55%, and about 27.6% of cloned embryos reached to hatching blastocyst stage with higher quality (about 92 cells compared to 116 cells, respectively) in the partial replacement group (P < 0.05). An explanation for this phenomenon might be related to the communication between embryo secretion factors to the in-vitro culture medium that played an essential role in embryo development (Wydooghe et al., 2017).

Table 4: Effect of Partial and Full Replacement (Changing Medium Methods) of Fresh mSOF Supplemented with 5% FBS to Cloned Bovine Embryos Culture Medium at Day 5 PA

Treatment	No. of Embryos Examined	No. (%) of Cloned Bovine Embryos Developed to			
		Morula	Early Blastocyst	Hatching Blastocyst	Cells Number
Full medium replacement	32	9 (28.1)[a]	7 (21.9)[a]	5 (15.6)[a]	92 (±.7.6)[a]
Partial medium replacement	29	16 (55.2)[b]	12 (41.4)[b]	8 27.6)[b]	116 (± 7.3)[b]

Transferring all cloned embryos into new drops (full replacement) may interrupt and remove the linkage of essential factors to embryos, thus affecting the development of embryos. Moreover, at the morula stage, the embryos were sensitive to the outside environment's change (Leibo et al., 1995). This issue affected some cloned embryos to stop developing at the morula stage after transferring into a new drop. Therefore, maintaining the embryo culture condition and replacing a part of the old medium (partial replacement) not only limit the effects of pipetting or shock

condition but still provide enough fresh medium and supplement (5% FBS) to stimulate the development of cloned bovine embryos to hatching blastocyst stage applied for further process to produce cloned cows.

The majority of cloned embryos did not develop to term, leading to the low overall cloning efficiency. Therefore, several research groups tried a variety of approaches to improve each step of the SCNT method. This study focused on optimization of cloning procedure in the in vitro culture system and achieved a high blastocyst formation rate comparing to the recent studies (Jang et al., 2011; Xu et al., 2019). To achieve the highest blastocyst development rate, the further research can use the combination of the procedure from this study with other methods like modifying DNA methylation and histone modifications, overexpression or suppression of embryonic-related genes, embryo transfer procedure and so on (Takahashi and First., 1992).

4. Conclusion

In conclusion, this report successfully established a culture system after SCNT performance to achieve a high percentage of viable cloned bovine embryos that can develop to the blastocyst stage. Using mSOF and changing the fresh mSOF medium with 5% FBS on day 3 following the partial replacement of fresh mSOF medium supplemented with 5% FBS on day 5 PA improved the developmental competence of cloned bovine embryos to blastocyst and promoted the blastocyst quality.

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