ESTABLISHMENT OF STORAGE METHOD FOR TRANSPORTATION OF CLONED BOVINE EMBRYOS BEFORE TRANSFERRING TO SURROGATE COW

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SUMMARY

Cloned bovine production has many practical applications, although the success rate remains low. Improving laboratory techniques have been investigated to overcome this problem. Embryo transportation has been considered to affect embryo survival rate and quality. Although the storage materials, such as plastic straws and microtubes, were studied in many species, there are few reports of cloned bovine embryos, and no paper compares the efficiency of different transportation systems. This study investigated the effects of transportation storage methods on pre-implantation development and the pregnancy rate of cloned bovine embryos. Briefly, each cloned bovine morula was stored in a plastic straw or a microtube without any hole or with two holes on its lid for two or four hours in the mini-incubator and then continued to culture in 5% CO_2 incubator until blastocyst formation. The results showed that cloned bovine embryos in microtubes with two holes maintained a higher rate of hatching blastocysts and average cell numbers after two- and four-hour incubation in the transportable mini-incubator. Furthermore, the pregnancy rate was higher for cloned bovine embryos stored in microtubes with two holes than the plastic straws after embryo transfer. In conclusion, transporting cloned bovine embryos in microtubes with two holes could enhance the pre-implantation development and pregnancy rate after embryo transfer.

Keywords: Cloned bovine embryo, transportation, pre-implantation development, pregnancy rate, plastic straw, microtube.

INTRODUCTION

Bovine is among the common livestock for animal cloning by somatic cell nuclear transfer, a process of introducing a nucleus of a donor cell into an enucleated oocyte. Although bovine cloning has many promising applications in basic research, reproductive cloning, and therapeutic cloning, its efficiency remains low, with the birth rate ranging from 5-15% (Gao *et al*., 2019). Many efforts have been made to enhance cloning efficiency, such as combining epigenetic modifiers to impair epigenetic errors of cloned embryos (Ding *et al*., 2008; Wang *et al*., 2011; Zhang *et al*., 2014).

Another approach is to improve the embryo transportation system, which can affect embryo survival and quality. Research led by Heyman *et al.*, 2002 emonstrated that pregnancy and live birth could be achieved when storing cloned bovine blastocysts at 39^{o}C in 0.25-mL plastic straws. The pregnancy rate on day 50 of gestation and birth rate were 27.1% and 6.8%, respectively (Heyman *et al*., 2002). Other transportation systems were similarly included in further studies, such as storing *in vitro* fertilization (IVF) bovine embryos in microtubes at 39^{o}C and 5% CO_2 in a transportable incubator (Morotti *et al*., 2014; Pontes *et al*., 2010). Later, a novel embryo transportation system was developed using punctured microtubes to facilitate gas exchange for storing and transporting IVF mouse embryos in a warm box at 38 ± 1^{o}C for four days (Tokoro *et al*., 2015).

Although there are many storage materials for embryo transportation, only one report mentioned the embryo transportation of cloned bovine embryos at warm temperatures (Heyman *et al*., 2002). Moreover, no paper has compared the effect of plastic straws and microtubes during transportation for embryo transfer. Therefore, this study aimed to determine the effects of transportation storage methods, namely microtube without holes, microtube with two holes, and plastic straw, on pre-implantation development and pregnancy rate of cloned bovine embryos.

MATERIALS AND METHODS

In Vitro Maturation

Bovine ovaries were collected from local slaughterhouses and transported to the laboratory within two hours. Then, large antral follicles (4-6 mm in diameter) were aspirated by an 18-gauge needle inserted into a 10-mL syringe

to collect oocyte cumulus-granulosa complexes (OCGCs). The complexes with intact, multilayered cumulus cells and homogenous dark cytoplasm were selected for *in vitro* maturation (IVM). Then, they were cultured in TCM-199 supplemented with 10% fetal bovine serum (FBS), 0.0065 IU/mL luteinizing hormone (LH), 1 mg/mL follicle-stimulating hormone (FSH), 50 ng/mL epidermal growth factor (EGF), 1 µg/mL 17β estradiol, and 50 µg/mL sodium pyruvate at 38.5^{o}C and 5% CO_2. After 18-22 hours of IVM, expanding cumulus cells were removed by 0.1% (w/v) hyaluronidase. Only mature oocytes with homogenous dark cytoplasm, spherical shapes, and the first polar body extrusion were chosen for producing cloned embryos.

Production of Cloned Bovine Embryos by Somatic Cell Nuclear Transfer

Fibroblasts from bovine (Hanwoo) lung tissue were used as donor cells, which were isolated and cultured in DMEM medium supplemented with 10% FBS, 0.07 mg/mL streptomycin, and 0.05 mg/mL penicillin at 38.5^{o}C and 5% CO_2. For somatic cell nuclear transfer (SCNT), cells from passages 3-10 were detached by 0.25% trypsin-EDTA to obtain a single-cell suspension and used as donor cells.

In the first step of SCNT, mature oocytes were enucleated in HEPES containing 7.5 µg/mL cytochalasin B, to which the MII spindle was positioned at three o'clock position with the first polar body below or nearby. The zona pellucida was opened using the XYClone® (Hamilton Thorne, USA) system, and the first polar body with a small amount of surrounding cytoplasm containing MII spindle was aspirated using an enucleation pipette. The MII spindle visible in the enucleation pipette confirmed the success of enucleation.

Then, the enucleated oocytes were washed and recovered in TCM-199 supplemented with 10% FBS and 50 µg/mL sodium pyruvate at 38.5^{o}C and 5% CO_2. For nuclear transfer, the fibroblast's nuclei were separated by gentle aspiration using an injection pipette to break the cell membrane. Each nuclear carried in the injection pipette was advanced inside the ooplasm, and the enucleated oocyte's membrane was broken with the assistance of MB-U piezo pulse (Prime tech, Japan) to expel the donor nuclear at nine o'clock position. After nuclear transfer, reconstructed oocytes were incubated for recovering until artificial activation.

At room temperature, the reconstructed oocytes were activated by 5 µM ionomycin for less than five minutes. Then, the oocytes were treated with 2 mM 6-DMAP and 250 nM scriptaid for four hours, followed by 250 nM scriptaid alone for 6 hours at 38.5^{o}C and 5% CO_2. Later, they were cultured in mSOF medium for *in vitro* development.

After the 52-hour activation, cloned bovine embryos were treated with preincubated 2.5 nM TSA and 125 nM scriptaid for 10 hours in the incubator. Finally, they were washed and cultured in mSOF medium supplemented with 5% FBS until they developed into the morula stage (Phuong *et al*., 2022)

Embryo Transportation Methods

Cloned bovine morulae were randomly stored in a microtube or a plastic straw. Each of the embryos stored in the microtubes was transferred to a 0.5-mL microtube without a hole or with two holes on its lid. A 20-gauge needle punctured each hole before loading 200-µL preincubated mSOF medium. For the embryos stored in the straws, each embryo was transferred to a 0.25-mL plastic straw (Sterile flexible 133mm mini-ET straw, cat. no. 006430, IMV Technologies, France). The following mixtures were loaded into the straw from left to right before sealing hermetically: mSOF medium, air bubble, mSOF medium, air bubble, mSOF medium containing one morula, air bubble, mSOF medium, air bubble, and mSOF medium.

For embryo transportation, both the microtubes and the plastic straws were placed in a mini-incubator (INC-RB1 BioTherm^{TM} transportable incubator, CryoLogic Pty. Ltd., Australia) at 38.5^{o}C for either two or four hours. After that, they continued to culture in the 5% CO_2 incubator until blastocyst formation. The developmental rate was calculated, and the cell numbers were determined by DAPI staining following fluorescent analysis by NIS-Elements BR Analysis 4.50.00 64-bit Program (Nikon Instruments Inc., Japan).

Embryo Transfer and Pregnancy Test

Sind-crossbred cows aged 2-5 years old were used as recipients for embryo transfer. The cows were first injected with 1.7 mL gonadorelin (Ovurelin, Bayer, New Zealand) on day 0, followed by 2.2 mL of cloprostenol (Ovuprost, Bayer, New Zealand) on day 7 and finally 1.7 mL of gonadorelin (Ovurelin, Bayer, New Zealand) on day 9 to induce estrus.

Cloned bovine embryos with good morphology were placed in microtubes with two holes or plastic straws and transported in the mini-incubator to the experimental farm. Each embryo was non-surgically transferred to the uterine horn of the synchronized surrogate cow. The total duration for transporting and preparing for embryo transfer was about four hours. Pregnancy was confirmed by a progesterone test on days 19-24 after embryo transfer.

Statistical Analysis

Each experiment was replicated at least three times. Statistical differences from the DAPI staining data were compared by one-way analysis of variance using IBM SPSS Statistics, version 25 (IBM Corp., Armonk, N.Y., USA). Results are presented as means. P values which are less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Effect of Transportation Methods on Pre-implantation Development of Cloned Bovine Embryos after Two Hours in the Mini-incubator

Each cloned bovine morula was stored in the mini-incubator. After that, the embryos were transferred to the 5% CO_2 incubator for further development. The pre-implantation development was recorded, which consisted of the developmental rate to hatching blastocysts and the average number of cells inside them (Figure 1 and Table 1).

In general, embryos stored in the mini-incubator for two hours had a lower rate of hatching blastocysts than those in the control group. The embryos in the microtubes with two holes (66.7%) and the plastic straws (66.7%) had a higher rate of hatching blastocysts than those in the microtubes without holes (25.0%) (Table 1). The reason behind this is that the change in extracellular pH influences the intracellular pH. When embryos were transported in the mini-incubator, which supplied heat only, a lack of CO_2 supplement resulted in the loss of CO_2 out of the bicarbonate-buffered mSOF medium, increasing the pH level. This phenomenon had been previously shown by Michl *et al.,* (2019) that the pH of the medium supplemented with 22 mM sodium carbonate could rise to about 7.8 and 8.3 after two and four hours outside the CO_2 incubator, respectively. Since mSOF medium contains 25.07 mM sodium bicarbonate, its pH may increase at a relatively same rate.

Consequently, the rise of extracellular pH would later affect the intracellular pH and cause stress to bovine embryos (Lane *et al*., 1999). Such a slight increase in intracellular pH could significantly inhibit glycolytic activity, which is vital for the metabolism of *in vitro* bovine morula and blastocyst (Gardner *et al*., 2015; Khurana *et al*., 2000; Rieger *et al*., 1992), impairing developmental competence (Gardner *et al*., 2015). Therefore, puncturing holes in the microtubes facilitated greater gas exchange through the medium, thus minimizing the pH shift over a period of time.

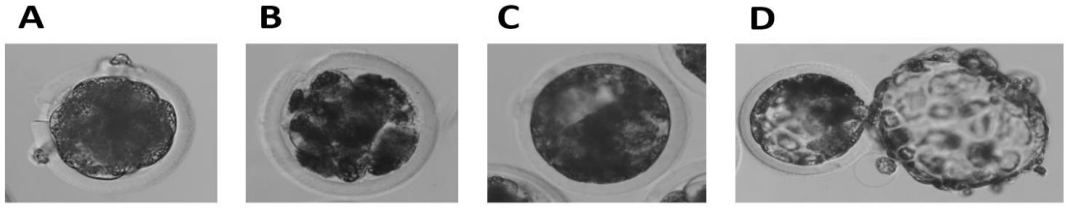

Figure 1. Pre-implantation development of cloned bovine embryo

(A) Cloned bovine morula before storing in the mini-incubator. After storing in the mini-incubator: (B) Arrested cloned bovine morula. (C) Early blastocyst of cloned bovine. (D) Hatching blastocyst of cloned bovine.

Table 1. Effect of transportation systems on pre-implantation development of cloned bovine embryos after two hours in the mini-incubator

Group	n	No. (%) of the embryo developed to		Average cell number of hatching blastocyst
		Early blastocyst	Hatching blastocyst	
Control	7	5 (71.4%)	5 (71.4%)	119.6
Plastic straw-2h	6	5 (83.3%)	4 (66.7%)	118.3
Microtube without hole-2h	8	6 (75.0%)	2 (25.0%)	116.8
Microtube with two holes-2h	6	5 (83.3%)	4 (66.7%)	119.1

n: Total number of examined embryos stored in mini-incubator. The embryos were at the morula stage before storage.

Control: The embryos were not stored in the mini-incubator.

Each experiment had at least three replicates.

Effect of Transportation Methods on Pre-implantation Development of Cloned Bovine Embryos after Four Hours in the Mini-incubator

Because the microtube without holes had a lower rate of hatching blastocysts than others, we stored embryos in plastic straws and microtubes with two holes in the mini-incubator for four hours. Similar to the previous finding, the rate of hatching blastocysts in the control group was higher than that of those stored in the mini-incubator. The microtube with two holes had a lower rate of hatching blastocysts after four hours than two hours, determining that intensive embryo transportation could affect embryonic development.

In addition, the rate of hatching blastocysts in the microtubes with two holes (54.5%) was much higher than that in the plastic straws after four hours (28.6%) (Table 2). Interestingly, the rate of hatching blastocysts in plastic straws remarkably decreased when stored in the mini-incubator for four hours compared with those for two hours.

The reason lies in the function of the plastic straw, which is for cryopreservation or embryo transfer. Therefore, it may have some limitations during embryo transportation, such as poor gas exchange or toxicity after prolonged storage at a warm temperature.

Apart from the developmental rate, we calculated each group's average cell number of hatching blastocysts. The average number of cells from the plastic straw after four hours was significantly lower than other groups (P<0.05) (Table 2). As a result, the microtube with two holes still maintained the pre-implantation development of cloned bovine embryos after four hours in the mini-incubator.

Table 2. Effect of transportation systems on pre-implantation development of cloned bovine embryos after four hours in the mini-incubator

Group	n	No. (%) of the embryo developed to		Average cell number of hatching blastocyst
		Early blastocyst	Hatching blastocyst	
Control	12	10 (83.3%)	8 (66.7%)	120.3[a]
Plastic straw-4h	7	6 (85.7%)	2 (28.6%)	113.4[b]
Microtube with two holes-4h	11	10 (90.9%)	6 (54.5%)	119.8[a]

n: Total number of examined embryos stored in mini-incubator. The embryos were at the morula stage before storage.

Control: The embryos were not stored in the mini-incubator.

a,b: Within the same column, values with the different superscript letters were significantly different (P<0.05). Each experiment had at least three replicates.

Effect of Transportation Methods on Pregnancy Rate of Cloned Bovine Embryos after Embryo Transfer

From the results above, we transported each cloned bovine morula in either the microtube with two holes or the plastic straw to the experimental farm, which took four hours for embryo transportation and preparation for embryo transfer. After transferring the embryo into each surrogate cow, pregnancy was confirmed by the progesterone test at days 19-24 of gestation.

The data showed that the pregnancy rate was much higher for embryos in the microtubes with two holes than those in the plastic straws, 54.5% and 25.0%, respectively (Table 3). Compared with the previous research, the pregnancy rate of microtube with two holes was similar to that at day 21 of gestation of somatic adult clones (55.6%) (Heyman *et al*., 2002). As a result, the microtube with two holes could maintain the developmental rate and blastocyst quality, thereby enhancing the pregnancy rate after embryo transfer.

Table 3. Effect of transportation systems on pregnancy rate of cloned bovine embryos after four hours in the mini-incubator

Group	No. of transferred embryos	Pregnancy rate
Plastic straw-4h	12	4 (25.0%)
Microtube with two holes-4h	11	6 (54.5%)

CONCLUSION

In conclusion, a microtube with two holes could store cloned bovine embryos for embryo transportation for up to four hours with significant pre-implantation development and a high pregnancy rate. Although the sample size was limited due to the objective obstacle of obtaining the cloned embryo transfer results and further research is therefore required, the results in this study have the potential to increase the cloning efficiency in not only bovine but also other species.

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THIẾT LẬP PHƯƠNG PHÁP BẢO QUẢN ĐỂ VẬN CHUYỂN PHÔI BÒ NHÂN BẢN TRƯỚC KHI CHUYỂN VÀO BÒ MANG THAI HỘ

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TÓM TẮT

Tạo ra bò nhân bản có nhiều ứng dụng thực tế tuy nhiên tỷ lệ thành công còn rất thấp. Cải thiện các kỹ thuật phòng thí nghiệm đã được nghiên cứu để khắc phục vấn đề này. Quá trình vận chuyển phôi có thể ảnh hưởng đến chất lượng và khả năng phát triển của phôi sau khi chuyển phôi. Mặc dù các vật liệu trữ phôi, cụ thể là ống rạ và ống ly tâm nhỏ, đã được nghiên cứu ở nhiều loài, nhưng có rất ít báo cáo về phôi bò nhân bản và không có bài báo nào so sánh hiệu quả của các hệ thống vận chuyển phôi khác nhau. Nghiên cứu này khảo sát ảnh hưởng của phương pháp lưu trữ đến sự phát triển tiền làm tổ và tỷ lệ mang thai của phôi bò nhân bản. Mỗi phôi dâu nhân bản được trữ trong ống rạ hoặc ống ly tâm nhỏ không đục lỗ hoặc đục hai lỗ trên nắp trong hai hoặc bốn giờ trong tủ cấy di động và sau đó tiếp tục nuôi cấy trong tủ ấm 5% CO_2 cho đến khi hình thành phôi nang. Kết quả cho thấy phôi bò nhân bản giữ trong ống ly tâm nhỏ có hai lỗ duy trì tỷ lệ phôi nang đang thoát màng và số lượng tế bào trung bình cao hơn sau hai và bốn giờ trong tủ cấy di động. Hơn nữa, tỷ lệ mang thai cao hơn đối với phôi bò nhân bản được lưu trữ trong ống ly tâm nhỏ có hai lỗ so với ống rạ sau khi chuyển phôi. Tóm lại, việc vận chuyển phôi bò nhân bản trong ống ly tâm nhỏ có hai lỗ có thể nâng cao sự phát triển tiền làm tổ và tỷ lệ mang thai sau khi chuyển phôi.

Từ khóa: Phôi bò nhân bản, vận chuyển phôi, phát triển tiền làm tổ, tỷ lệ mang thai, ống rạ, ống ly tâm nhỏ.

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