# Production of Cloned Calves by the Transfer of Somatic Cells Derived from Frozen Tissues Using Simple Portable $CO_2$ Incubator*

**Y. J. Dong, X. J. Bai, M. D. Varisanga[2], N. R. Mtango[1], T. Otoi[1], R. Rajamahendran[3] and T. Suzuki**[**,1]**
Faculty of Animal Science, Raiyo Agricultural College, China

**ABSTRACT :** The ability of frozen-thawed fetal skin was examined to generate viable cell lines for nuclear transfer. Fetal skin frozen at -20°C, -30°C or -80°C in the presence of 5% DMSO used as tissue explants to generate somatic cells. The resultant confluent cells were then used as donors for nuclear transfer (NT). Of the bovine NT embryos reconstracted from the somatic cells, 62.3%, 76.6% to 65% showed cleavage 70.5%, 81.9% to 78.5% reached the stage of morula formation and 39.7%, 43.2% or 47.6% reached the blastocyst stage. There was no significant difference in development when the NT embryos were compared with those reconstracted from fresh somatic cell derieved skin tissues (72%, 75.3%, and 45.2%, for cleavage, and development to morula and blastocyst stage, respectively). NT embryos were then placed in a portable $CO_2$ incubator and carried to China from Japan by air. After reaching to farm, two NT embryos were transferred to each of 5 recipients. We obtained 2 NT calves which birth weights is 30kg and 36kg female, and gestation periods is 281 and 284 days, respectively. There were no observation any abnormality from those calves. The results indicated that cell lines derieved from bovine fetal skin cryopreserved by a simple method could be used as donors in nuclear transfer using the portable $CO_2$ incubator. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 2 : 168-173)***

**Key Words :** Cloning, Frozen Fetal Skin, Electrofusion, Portable $CO_2$ Incubator, Blastocysts, Calves

## INTRODUCTION

The successful production of offspring from differentiated somatic cells by nuclear transfer techniques has demonstrated that the nuclei of these cells can be reprogrammed by the cytoplasm of recipient oocytes (Campbell et al., 1996; Kato et al., 1998; Wakayama et al., 1998; Ogura et al., 2000). These successes have opened wide opportunities to produce transgenic animals and elite livestock from adult animals, and to conserve threatened species. Fresh and frozen-thawed somatic cells have been used successfully in nuclear transfer to produce offspring (Wilmut et al., 1997; Cibelli et al., 1998).

Cryopreservation techniques have been widely used to preserve cell lines or embryonic cells for research or breeding purposes. In cloning by nuclear transfer in domestic animals, cell lines derieved from adult or fetal tissues have been widely used to prepare donor cells, and subsequent cell lines have been frozen for other purposes. However, with all of the above techniques, special care and preparation must be used to maintain the viability of field samples until they are subjected to further steps in the laboratory.

The first aim of this study was to explore the possibility of generating somatic cell lines from tissue explants that had been frozen and kept for a period of time. The second aim was to examine the viability of the resultant cells by transferring them to enucleated matured oocytes. The third aim was to obtain the calves after transfer to recipients.

On the other hand, since pre- and peri-natal abnormality and mortality have been observed in both *in vitro* embryo production and nuclear transfer of somatic cells, it can be said that the current *in vitro* culture conditions have not been yet fully optimized. It is therefore important to consider the cultural environment when one is attempting to improve an embryo culture system. In a previous study (Varisanga et al., 2000; 2002), we found that reconstructed bovine blastocyst could be obtained by using a simple portable $CO_2$ incubator that differed from standard incubators in that embryos were grown under the influence of negative air pressure. Additionally, the incubator chamber designed by Suzuki and co-workers (Suzuki et al., 1999) can be used to study the effects of alterations in oxygen and carbon dioxide levels, and air pressure on embryo development. In the present study, we used portable incubator for reconstructed cloning embryos and carried to China from Japan, then transferred these embryos to synchronized recipients.

## MATERIALS AND METHODS

### Description of the incubation chamber

In this experiment, a simple portable $CO_2$ incubator

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** Corresponding Author: T. Suzuki. Fax: +81-82-273-4323, E-mail: suzuki1939@jasmine.ocn.ne.jp
[1] The United Graduate School of Veterinary Sciences, Yamaguchi University, Laboratory of Animal Reproduction & Applied Biotechnology, 1677-1 Yoshida, Yamaguchi 753-8515, Japan.
[2] Faculty of Science, Technology and Environmental Studies, The Open University of Tanzania, Dar es Salaam, Tanzania.
[3] Departments of Animal Science, The University of British Columbia, Canada.
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(Fujihira Industry Co. Ltd., Japan) consisting of an insulated metal box (29×26×21 cm for L×W×H, respectively, and 15.8 L volume) with a thermostat and temperature gauge was electrically heated to maintain a chamber temperature of 38.5°C. All incubations assigned to the portable incubator (*in vitro* maturation and culture of the reconstituted embryos) were performed in a small plastic box (chamber box: 15×10×4.5 cm=0.68 L volume) placed inside. After being covered to make airtight, this chamber box was given a negative air pressure (-300 mm Hg) by aspiration of the inside air with a hand vacuum-pump (Fujihara Industry Co. Ltd., Japan). Carbon dioxide gas was produced by adding 5 ml of distilled water to 0.35 g of effervescent granules (EG: 420 mg tartaric acid, 460 mg $NaH_2CO_3$ and 10 mg silicone fiber per gram; Horii Pharmaceuticals Co., Osaka, Japan) in a small Petri dish (diameter×height=34×20 mm) to release about 5% $CO_2$ and 8 to 10% $O_2$.

**Oocyte collection and *in vitro* maturation**

Bovine ovaries (Japanese Black) were collected from a local abattoir and transported to the laboratory within 2 h of collection (kept at 30°C) in saline solution (0.85% NaCl) containing 200 IU/ml penicillin G potassium and 0.2 g/l streptomycin sulfate. The ovaries were washed three times with fresh saline solution at arrival, and the contents of small non-hemorrhagic follicles (2 to 6 mm in diameter) were aspirated with an 18 g needle attached to a 10 ml syringe. Follicular fluid was washed from the cumulus oocyte complexes (COCs) with modified phosphate-buffered saline (mPBS) solution. COCs that had a complete unexpanded cumulus and a cytoplasm that appeared homogenous were selected, washed three times in maturation medium and cultured. Oocytes was cultured for 20 to 21 h at 38.5°C in droplets (20 to 30 oocytes per 100 μl drop) of maturation medium covered with mineral oil (E. R. Squibb & Sons, Inc. NJ, USA) and placed in a plastic container in the portable chamber box under 5% $CO_2$, 8% to 10% $O_2$ atmosphere and -300 mm Hg air pressure. The maturation medium used consisted of Synthetic Oviduct Fluid (SOF) was supplemented with 1% basal medium Eagle-essential amino acid (BME-EAA, Sigma, St. Louis, MO, USA), 1% minimum medium-nonessencial amino acid (MEM-NEAA, Sigma), 5 mM taurine (Sigma), 0.5 mM pyruvic acid (Sigma), 1 mg/ml polyvinyl alcohol (PVA, Sigma), 0.455 mg/ml alanine (Sigma), 0.146 mg/ml glutamine (Sigma), 0.375 mg/ml glycine (Sigma), 5% heat-treated fetal cow serum (FCS; JRH Biosciences Lenaxa, KS, USA), 10 IU/ml follicle stimulating hormone (FSH; Denka Pharmaceuticals, Kawasaki, Japan), and 50 μg/ml gentamicin.

**Recipient oocytes**

Following *in vitro* maturation, oocytes were stripped free of the surrounding granulosa and cumulus cells by treating them with 0.5% hyaluronidase (Sigma) for 2 min, followed by repeated pipetting with a narrow-bore micropipette in mPBS. Oocytes with the first polar body extruded were selected as recipient cytoplasts. They were placed into mPBS containing 3 mg/ml bovine serum albumin (BSA; Sigma) and 5 μg/ml cytochalasin B (C.B.; Sigma). The zona pellucida of the recipient oocyte was cut by a sharp glass needle before enucleation. Enucleation of the metaphase plate chromosome was accomplished by microsurgical enucleation of the first polar body and a small amount of the cytoplasm adjacent to the first polar body, using a 35 μm non-beveled glass pipette. Successful enucleation was confirmed by exposing all the removed segments to ultraviolet light and checking for the presence of a metaphase plate stained with 5 μg/ml Hoechst 33342. Enucleated oocytes were then washed three times in a CB-free medium. They were then ready for nuclear transfer.

**Donor cells**

A fetus (of approximately 8 months., F1 cross: Holstein×Japanese Black) obtained at a local slaughterhouse was carried to the laboratory in physiological saline (0.9% [w/v] NaCl) at 30 to 37°C within 3 h after slaughter. After the fetus had been washed three times in the saline solution, the skin was carefully separated from the underlying tissues, then cut into pieces (0.5 to 1 $cm^2$) and placed into several 50 ml conical tubes (Falcon, USA.) filled with Dulbecco's phosphate buffered saline solution (Gibco BRL, Life Technologies, USA) supplemented with 5% (v/v) dimethylsulfoxide (DMSO, Wako, Japan). The tissues were kept in this solution at room temperature for 20 min, then put into freezer at -20, -30 or -80°C for about 3 months before further use. As negative controls, some of the tissues were put into PBS without the addition of DMSO, and were then frozen at -20, -30 or -80°C. As positive control, some of the tissues were put into PBS for washing and cultured in modified D-MEM for 5 to 8 days before use.

Thawing was accomoplished by warming the conical tube in a dry thermo unit (DTU-IB, Taitec Co., Japan) at 37°C until the ice had completely melted. The skin was minced into small pieces in fleshly prepared PBS, and then subjected to enzymatic digestion with 0.25% trypsin-EDTA in Tris-saline solution for 30 min at 38.5°C. The skin was then washed with PBS by centrifugation at 3,000 rpm×5 min, twice. The final tissue pellet was cultured in Dulbecco's modified Eagle's medium (D-MEM; Gibco) supplemented with 5% fetal bovine serum (FBS) and 50 μg/ml gentamicin suffate (Sigma) in a 35 mm cell culture

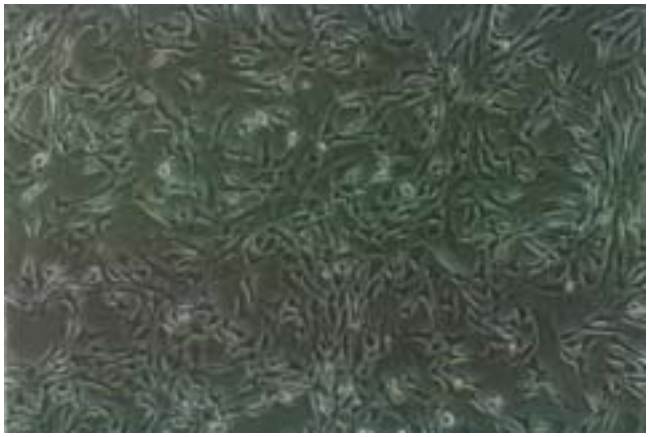

**Figure 1.** Monolayer of somatic cell lines after culture in DME+5%FBS.

dish (Falcon, USA) at 38.5°C under a humidified atmosphere of 3.5% $CO_2$ in air. Figure 1. show the donor cells used in this experiment.

**Nuclear transfer**

The donor cells used for NT were between 4 and 8 passages of culture, and they were synchronized in presumptive $G_0$ by serum deprivation (i.e., cultured in D-MEM plus 0.5% FCS for 5 to 8 days before use). Immediately before injection, a single cell suspension of donor cells was prepared by standard trypsinization. Oocytes were reconstructed by inserting the donor cell into the perivitelline space of a recipient oocyte through the slit in the zona pellucida made during the enucleation. The cells were wedged between the zona and the cytoplast membrane to facilitate membrane contact before fusion. The reconstructed oocytes were incubated in fusion medium [0.3 M manitol+0.1 mM $CaCl_2$+0.1 mM $MgSO_4$+2 mg/ml fatty acid-free BSA (Sigma)] for 5 min before electrofusion. The single cell-oocyte complex was kept between the electrodes and oriented with the contact surface between the cytoplast and the donor cell perpendicular to the electrodes. Cell fusion was induced with a single DC pulse of 700-800 V/cm for 50 μsec with an ECM2001 (BTX; San Diego, CA, USA) electro cell manipulator. Following the fusion pulse, the complexes were washed in mSOF medium containing 10 μg/ml insulin, 1 mg/ml PVA, 3 mg/ml BSA and cultured for at least 1 h in 100 μL of the same medium before chemical activation. Fusion was then determined by microscopic examination. Those reconstructed oocytes that had fused were then treated with Ca ionophore A 23187 (10 μM; Sigma) for 5 min followed by cyclohexamide (10 μg/ml; Sigma) for 4 h.

***In vitro* culture of the reconstructed embryos**

Reconstructed couplets (10 to 15) were transferred to 100 μl of mSOF medium and cultured for an additional 8 days to assess their ability to develop into blastocysts. After 48 h of culture, the embryos were examined for cleavage and picked up to prevent them from sticking to the bottom of the culture drop. Un-cleaved embryos and/or degenerated ones were also removed from the culture.

**Embryo transfer and survival evaluation**

Recipient cows (Holstein) were synchronized with a single 10 day EAZI-BREED™ (CIDR-plus; Inter Ag, Hamilton, New Zealand) treatment. After the CIDR-plus had been in place for 6 d, each cow received 0.526 mg chlorprostenol (2 ml estrumate; Sumitomo Pharmaceutical Co., Japan). The onset of estrous was observed 48 h after CIDR-plus withdrawal, and embryo transfer was carried out on Day 7 (estrus=day 0, the same day which fusion was performed). The developmental stage of reconstructed embryos was compacted morula on Day 6 at the start of transportation in Japan, and blastocyst on day 7 at transfer into recipients in China. Ten embryos (blastocyst) were transferred to five synchronized recipients (i.e., two embryos per recipient) at Laiyang College of Agriculture, China.

The recipients were examined by ultrasonography (ECHO VISION SSD-500EV monitor with a linear 5 MHz rectal probe; all from Aloka Co. Ltd., Japan) on Days 20, 40, 60, 90 and 120 to record fetal development or survival rates following embryo transfer.

**Statistical analysis**

A total of 5 replications for each incubation group were conducted. All percentage data were subjected to arcsine transformation, and the data for cleavage and developmental rates were expressed as percentages. Developmental rates between experimental groups were analyzed by Student's *t*-test and Duncan's multiple range tests using the General Linear Models procedure of the Statistical Analysis System. Probability of $p<0.05$ was considered to be statistically significant.

## RESULTS

The results of culture of cells obtained from the skin frozen with DMSO survived at -20, -30 and -80°C, however there was not detected survive from the skin frozen without DMSO.

Of the bovine NT embryo reconstructed from the somatic cells frozen at -20, -30, -80°C in the presence of 5% DMSO, 62.3, 76.6 to 65% showed cleavage 70.5, 81.9 to 78.5% reached the stage of morula formation and 39.7, 43.2 to 47.6% reached blastocyst formation. There were no significant differences in development when the NT embryos were compared with those reconstructed from fresh somatic cell derieved skin tissues (72, 75.3 and 45.2%,

**Table 1.** The *in vitro* development of bovine cloned embryos derived from frozen-thawed tissues as donor nuclear

| Donor cells | No. of reconstructed embryos (replicate) | Cleavage (%) | Development (%) to | |
|---|---|---|---|---|
| | | | Morulae | Blastocyst |
| * F-NT-20 | 109 (4) | 68 (62.3) | 48 (70.5) | 27 (39.7) |
| F-NT-30 | 145 (6) | 111 (76.6) | 91 (81.9) | 48 (43.2) |
| F-NT-80 | 100 (4) | 65 (65.0) | 51 (78.5) | 31 (47.6) |
| ** NF-NT | 128 (6) | 93 (72.7) | 70 (75.3) | 42 (45.2) |

* F: Frozen, ** NF: Non frozen.

**Table 2.** Result of bovine cloned embryos transfer which cells obtained from the skin frozen at -30°C

| No. of recipients transferred | No. of pregnancies (%) | * No. of fetus (%) | ** No. of abortion (%) | No. of delivery (%) |
|---|---|---|---|---|
| 5 | 3 (60) | 3 (60) | 1 (20) | 2 (40) |

* Fetus was observed on days 20, 40 and 60 using ultrasonography. **Abortion was detected on days 60 after transfer.

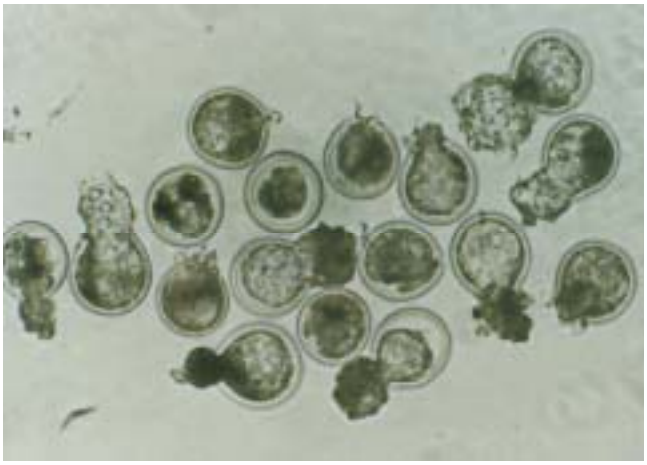

**Figure 2.** Day 7 reconstituted bovine blastocysts following nuclear transfer obtained from skin frozen at -30°C *in vitro* culture.

for cleavage, and development to morula and blastocyst stage, respectively). (Table 1, Figure 2)

We obtained 2 NT calves which were transferred with NT embryos reconstructed from skin frozen at -30°C, their birth weight was 30 and 36 kg, and gestation period was 281 and 284 days, respectively. (Table 2 and Figure 3). There were no observation of any abnormality from those calves and DNA on fetal skin-frozen and blood on calves were corresponded by microsatellite genotyping analysis (Figure 4).

## DISCUSSION

In the negative controls, no cell lines had been established after 2 weeks of cell culture. However, skin frozen at -20, -30 or -80°C in the presence of DMSO formed tissue clumps surrounded by a cell layer after 10 to 14 days of culture. This result suggests that the presence of DMSO as a cryoprotectant is essential to maintain the

**Figure 3.** Two calves (F1 cross with Holstein and Japanese Black) was obtained after transfer of NT embryos.

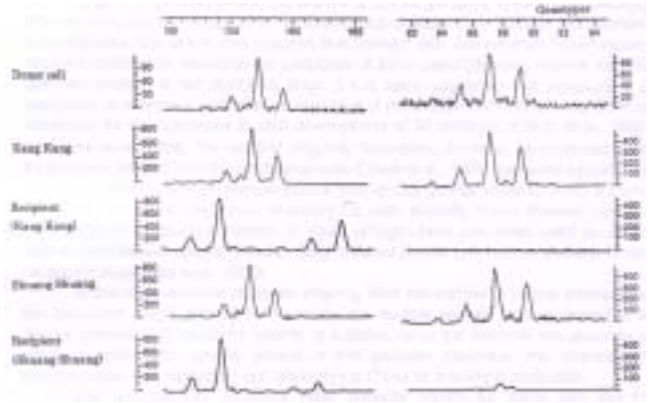

**Figure 4.** DNA on fetal skin frozen and blood on calves were corresponded by microsatelite genotyping analysis using 13 marker.

viability of skin tissues subjected to cryopreservation. Morphological observation suggested that some parts of the tissues showed evidence of degeneration after thawing and culture, but the formation of clumps in the center of the cultured tissue suggested that some cells survived when the tissue was frozen in the presence of cryoprotectant, and these provided cell lines when the tissue was subjected to further culture (Figure 1). Mazur (1977) reported that the survivability of cryopreserved cells was dependent on the concentration of cryoprotectant. High concentrations of DMSO gave higher rates of cell survival at temperatures

from below 0°C to -80°C. In mice, offspring have been produced from oocytes injected with spermatozoa that have been cryopreserved at -50°C with or without cryoprotectant (Wakayama et al., 1998). Also, cryopreservation of ovarian tissues followed by the use of allografting and xenografting techniques has been reported to be successful in restoring oocyte fertility (Godsen et al., 1994; Cox et al., 1996; Gunasea et al., 1997) and producing offspring (Liu et al., 2001).

The *in vitro* development of NT embryos reconstructed from somatic cells derived from frozen or unfrozen fetal tissue is summarized in Table 1. The percentages of cleavage and development to the morula or blastocyst stage were not significantly different ($p>0.05$) from those of NT embryos reconstructed from somatic cells derived from unfrozen fetal tissue. This suggests that somatic cells derived from frozen tissues have the ability to be altered by the cytoplasm of the oocytes following nuclear transfer and can develop to the blastocyst stage. It has been suggested that exposure to a cytoplasm environment containing a high level of maturation promoting factor (MPF) is necessary for the successful *in vitro* development of NT embryos (Collas et al., 1992; Campbell et al., 1994). The fact that offspring have been shown to be produced from NT embryos derived from frozen-thawed cells (Cibelli et al., 1998) opens the opportunity for us to conduct long-term preservation of high-quality genetic material in the somatic cell from much more simply than as embryonic cells. Recently, frozen-thawed cumulus cells stored in mechanical freezers or liquid nitrogen have also been used as donor cells in somatic cell nuclear transfer. They showed similar potential to develop to the blastocyst stage (Tani et al., 2000).

In this study we have obtained offspring from the transfer of bovine embryos (at the blastocyst stage) derived from somatic cell nuclear transfer and incubated in a simple, portable $CO_2$ incubator system. In addition, since the embryos are grown in a small chamber box usually placed in the portable incubator, the chances of transportation was easily from our laboratory to China for transfer to recipients.

The portable $CO_2$ incubator gave superior results for these two indices compared with those obtained from the standard $CO_2$ incubator (Varisanga et al., 2000, 2002). Given the fact that the same reconstruction protocol was employed in both systems, the negative air pressure, which was present in the portable incubator, may have contributed positively towards the developmental ability of the reconstituted couplets. The precise mechanism is not yet clear, but as the $O_2$ tension was lowered substantially during the creation of negative air pressure, fewer free oxygen radicals which are considered to damage the mitochondria (Menke and McLaren, 1970) were left available. In fact, it recently been hypothesized that free oxygen radicals causes a loss of intracellular pH (pHi) regulation, which may alter mitocondrial functions such as phosphorylation and energy metabolism (Bavister, 2000), resulting in early embryonic loss, perturbed growth and/or anomalies in offspring. Another possible means by which the negative air pressure could exert its influence on the growth and developmental competence of the embryos would be by making it mechanically easier for the number of cells to increase during mitotic division of the reconstituted couplets.

The survival rate of reconstituted embryos from the portable incubator 40 days after transfer, as indicated by ultrasonography, was relatively high (60%) in this study. Nevertheless, embryonic loss before or by Day 120 was comparatively high (40%), in contrast with 30% for bovine IVP embryos (McMillan et al., 1996). There are various reports suggesting that the early embryonic mortalities observed in IVP and clone embryos are associated with both maternal anatomical failure such as retardation in allantois development and lack of vascularization (Peterson et al., 1998), or a low number of placentomes (causing placental dysfunction) (Arthur et al., 1989) and fetal losses during the middle of the first trimester as consequence of hydrallantois (Wells et al., 1999), enlarged umbilical vessels, and edematous placental membranes (Kruip and Den Daas, 1997; Cibelli et al., 1998; Van Wagtendonk Leeuw et al., 1999). However, there were no observed such as abnormalities from offspring.

The use of our simple portable $CO_2$ incubator with negative air pressure is an alternative method for the production of cloned bovine embryos (Kruip and Den Daas, 1997; Wells et al., 1999) and the establishments of pregnancies by the somatic cell nuclear transfer technique. We have identified a new element in the culture conditions-negative air pressure- that has proven to be useful for this cloning protocol. However, as for any other method of nuclear transplantation, the culture conditions alone are not the only factors that contribute to the low efficiency currently obtained with this new technology. Other factors, such as the cell cycle of the recipient cytoplasm, the activation protocol, a better understanding of the reprogramming of differentiated nuclei, and the focus on the development and function of the placenta needs to be considered. We are intending to carry out further studies with the portable incubator system to determine the optimal levels of $O_2$, $CO_2$ and air pressure for early embryo development *in vitro*.

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