Cloned Porcine Embryos can Maintain Developmental Ability after Cryopreservation at the Morula Stage

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Abstract. The aim of the present study was to clarify the overall efficiency of porcine somatic cell nuclear transfer (SCNT) by incorporating cryopreservation of the cloned embryos before transfer. The SCNT embryos reconstructed with preadipocytes and in vitro-matured (IVM) oocytes were cultured to harvest morula stage embryos; they were then subjected to delipiation (removal of cytoplasmic lipid droplets) and vitrification. After warming and culture, the embryos developing to blastocysts were transferred to recipients to obtain cloned piglets. From 372 reconstructed embryos, 188 (50.5%) reached the morula stage and 117 (31.5%) developed to blastocysts after vitrification. Transfer of 98 (26.3%) morphologically normal blastocysts gave rise to 6 (1.6%) piglets, including 1 stillborn. The efficiency of the cloned piglet production was comparable with that obtained using SCNT embryos without cryopreservation (2.7%, 17/98 (26.3%) morphologically normal blastocysts gave rise to 6 (1.6%) piglets, including 1 stillborn). The efficiency of the embryos, 188 (50.5%) reached the morula stage and 117 (31.5%) developed to blastocysts after vitrification. Transfer of 98 (26.3%) morphologically normal blastocysts gave rise to 6 (1.6%) piglets, including 1 stillborn. The efficiency of the cloned piglet production was comparable with that obtained using SCNT embryos without cryopreservation (2.7%, 17/635). Here, we demonstrate that porcine somatic cell cloning can be performed without a significant reduction in efficiency even when the SCNT embryos are cryopreserved before transfer.

Key words: Pig cloning, Somatic cell nuclear transfer, Vitrification

In recent years, pigs have often been used as large laboratory animals in biomedical research [1-7], and genetically engineered pigs are highly desired in rapidly advancing research areas, such as regenerative medicine, organ transplantation, analysis of intractable genetic disorders and stem cell therapy [for review, see 7]. For the production of genetically engineered pigs, somatic cell cloning is a key technology [8-15]. Therefore, if a reliable cryopreservation technique for cloned embryos can be established, the production and application of various specially designed pigs will advance further.

We conducted the present study to elucidate the conditions that could achieve a high survival rate of cloned porcine embryos after cryopreservation in a stable manner. We previously showed that, for the cryopreservation of porcine IVM-derived embryos, it is effective to combine vitrification and delipiation, a process for reducing the amount of lipid droplets in the embryonic cytoplasm [16, 17]. We have also confirmed in our previous studies that delipiation of porcine embryos at the morula stage is less injurious than when done at the early cleavage stages of the blastocyst stage [16-19]. In addition, it was shown that vitrification of delipated porcine embryos at the morula stage results in a high postwarming survival rate [16, 18, 19]. In conjunction with these studies, we carried out a study of the vitrification of porcine cloned embryos at the morula stage. The overall efficiency of cloned pig production by SCNT with cryopreservation (i.e., how many cloned piglets could be produced from all of the SCNT embryos prepared via cryopreservation) was clarified.

In the cryopreservation experiment, the SCNT embryos reconstructed with preadipocytes [20, 21] and IVM oocytes were cultured to allow development to the morula stage, and they were then subjected to delipiation and vitrification by the Minimum Volume Cooling (MVC) method [22]. After warming, the recovered embryos were cultured, and those developing to blastocystcs were transferred to recipients in order to obtain cloned piglets (Table 1). Of the 372 SCNT embryos produced, 188 (50.5%) developed in vitro into morphologically normal morulae (Fig. 1a). All of these embryos were then subjected to delipiation and vitrification. After warming, 172 embryos (46.2% ; Fig. 1b) were recovered. Sixteen embryos were lost during vitrification or warming. The recovered embryos were cultured, and 117 (31.5%) embryos developed to the blastocyst stage. Of these, 98 (26.3%) morphologically normal blastocysts (Fig. 1c) were selected and transferred to two recipient gilt (47 and 51 embryos per recipient). Both recipients became pregnant, and a total of six piglets (1.6%) were obtained (Fig. 1d). Because one piglet was stillborn, the overall efficiency of live piglet production was 1.3% (5 live piglets out of 372 SCNT embryos produced). These data clearly showed that the combination of delipiation and vitrification is effective for cryopreserving porcine SCNT embryos.

For comparison, Table 1 presents results of an experiment in which cloned embryos derived from the same nuclear donor cells
313 VITRIFICATION OF PORCINE CLONED EMBRYOS were transferred without cryopreservation to recipient pigs. In this experiment, a total of 635 SCNT embryos were prepared, and after 24–48 h of culture, 400 (63.0%) morphologically normal embryos at the 1–2-cell (day-1) or 2–8-cell (day-2) were transferred to four recipient pigs (77–123 embryos per recipient). All of the recipients became pregnant, and a total of 17 (2.7%) cloned piglets, including 7 stillborn, were obtained. Thus, cloned piglets could be obtained from cryopreserved SCNT embryos without a critical decrease in efficiency (2.7 vs. 1.6%, not a significant difference).

In the present study, the cloned embryos prepared without cryo-

Table 1. Production efficiency of cloned piglets from cryopreserved or noncryopreserved SCNT embryos

<table>
<thead>
<tr>
<th>Cryopreservation</th>
<th>SCNT Embryos Produced</th>
<th>Developed to the Morula Stage (%)</th>
<th>Successfully Delipated and Vitrified (%)</th>
<th>Recovered and Cultured (%)</th>
<th>Developed to the Blastocyst Stage (%)</th>
<th>Transferred to Recipients (%)</th>
<th>Cloned Piglets Delivered (%)</th>
<th>Live Offspring (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>372</td>
<td>188 (50.5)</td>
<td>188 (50.5)</td>
<td>172 (46.2)</td>
<td>117 (31.5)</td>
<td>98 (26.3)</td>
<td>6 (1.6)</td>
<td>5 (1.3)</td>
</tr>
<tr>
<td>–</td>
<td>635</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>(63.0)</td>
<td>17</td>
<td>10 (1.6)</td>
</tr>
</tbody>
</table>

* Percentage as a portion of the total number of SCNT embryos produced. Cloned embryos were transferred to the uterine horns at the blastocyst stage or to the oviducts at the early cleavage stage.

![Fig 1](image1.png)

Fig 1. Morphological appearance of porcine cloned embryos before and after cryopreservation, and cloned piglets developed from the vitrified embryos. a: SCNT embryos developed to the morula stage before delipation and vitrification. b: An SCNT morula recovered after delipation and vitrification. c: Day 6 blastocysts developed from vitrified SCNT morulae. d: Piglets produced from cloned embryos vitrified at the morula stage. Scale bars=100 μm.
preservation were transferred at the early cleavage stage (days 1–2), avoiding a prolonged culture that may have an adverse effect on the viability of embryos. This timing for transfer of the noncryopreserved SCNT embryos is routinely used as the most feasible condition for cloned pig production in our laboratory [14, 15, 21, 23]. Furthermore, noncryopreserved embryos were transferred to sexually mature pregnant pigs that had been artificially aborted, while gilt embryos were used as the recipients for transferring the cryopreserved embryos. Compared with prepubertal gilt recipients, the uterus of sexually matured gilts is more developed, and this could have contributed to the better development of the transferred cloned embryos. Considering all these circumstances, the fact that cloned piglets could be obtained from cryopreserved SCNT embryos without a critical decrease from the efficiency obtained with the noncryopreserved embryos (1.6 vs. 2.7%, not a significant difference) would have a significant impact on the practical application of pig cloning technology, including the production of genetically modified pigs.

The previous reports on cryopreservation of SCNT embryos [24–26] did not clarify the difference in the efficiency of cloned pig production with or without cryopreservation, nor did they show whether a marked decrease in efficiency was seen with cryopreservation. Therefore, the practical advantage of cryopreserving porcine SCNT embryos remained to be clarified. The present study clarified the following points: 1) the actual proportion of prepared cloned embryos that can be cryopreserved; 2) the proportion of cryopreserved embryos that survive after thawing and can be used for transfer; and 3) the efficiency of cloned pig production in relation to the total number of SCNT embryos prepared with cryopreservation. Particularly with regard to the third point on the efficiency of cloned pig production, a comparison was made with SCNT embryos derived from the same donor cells without cryopreservation. The dissociated cells were filtered through a 100-μm nylon mesh (Falcon 352360; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and centrifuged at 150 × g for 5 min. After centrifugation, only the mature adipocytes that were floating near the surface of the supernatant were collected. These cells were washed several times, and approximately 10^6 of these cells were placed in a 12.5-cm² culture flask (Falcon 353018; Becton, Dickinson and Company) filled with DMEM containing 20% (v/v) fetal bovine serum (FBS; SAFC Biosciences Inc., Kansas City, KS, USA). The flask was brimmed with medium and tightly capped, providing the adipocytes with an air-free environment. The flask was incubated upside down in a humidified atmosphere of 5% CO₂ in air at 37°C so that the floating adipocytes attached to the inner ceiling surface of the flask. The cells were cultured for 7 days, and they underwent a morphological change to a fibroblast-like shape. An accompanying gradual decrease in the amount of cytoplasmic lipid droplets was confirmed in those cells attached to the flask ceiling. When firm attachment of the cells to the ceiling surface of the flask was confirmed after 7 days, the flask was turned upside down and cultured using the routine cell culture technique for adherent cells. The medium was changed every 3 days. After 3 passages, the preadipocytes were suspended in medium with 10% (v/v) dimethyl sulfoxide (DMSO; Nacalai Tesque, Inc., Kyoto, Japan) and frozen in liquid nitrogen.

SCNT was performed using IVM oocytes as the recipient cytoplasm as described previously [14, 21]. Enucleation was performed using a chemically assisted method developed by Yin et al. [29]. The oocytes were cultured in NCSU23 medium supplemented with 0.1 μg/ml demecolcine, 0.05 M sucrose (Nacalai Tesque) and 0.4% (v/v) BSA for 0.5–1 h. When a protrusion was observed on the surface of an oocyte, it was removed along with the polar body using a beveled pipette (30 μm in diameter) in HEPES-TL-PVP containing 0.1 μg/ml demecolcine, 5 μg/ml cytochalasin B (CB) and 10% (v/v) FBS. Enucleation of the oocytes without the protrusion was carried out by removing the polar body and a small amount of the adjacent cytoplasm (less than 10%).

Methods

Chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise indicated. Ovaries were collected at a local abattoir and transported to the laboratory in Dulbecco’s phosphate buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan) containing 75 μg/ml potassium penicillin G, 50 μg/ml streptomycin sulfate, 2.5 μg/ml amphotericin B and 0.1% (w/v) polyvinyl alcohol (PVA). Cumulus-oocyte complexes (COCs) were collected by aspiration from ovarian antral follicles with a diameter of 3.0–6.0 mm. Those COCs having at least three layers of compacted cumulus cells were selected and cultured in NCSU23 medium [27] supplemented with 0.6 mM cysteine, 10 ng/ml epidermal growth factor (EGF), 10% (v/v) porcine follicular fluid, 75 μg/ml potassium penicillin G, 50 μg/ml streptomycin sul-
Primary culture cells were used as nuclear donors after cell cycle synchronization by serum starvation (0.5% (v/v) FBS) for 48 h. A single donor cell was inserted into the perivitelline space of an enucleated oocyte. The donor cell-oocyte complexes were placed in a 280-mM mannitol (Nacalai Tesque) solution (pH 7.2) containing 0.15 mM MgSO₄, 0.01% (w/v) PVA and 0.5 mM Hepes, and they were held between two electrode needles. Membrane fusion was induced with a somatic hybridizer (SSH-1; Shimadzu, Kyoto, Japan) by applying a single direct current (DC) pulse (200 V/mm, 20 μsec) and a pre- and postpulse alternating current (AC) field of 5 V and 1 MHz for 5 sec, respectively. The reconstructed embryos were cultured in NCSU23 for 1-1.5 h, followed by electrical activation with a single DC pulse of 150 V/mm for 100 μsec. For activation, the fusion solution described above was modified to contain 0.1 mM MgSO₄ and 0.05 mM CaCl₂. The activated embryos were treated with 5 μg/ml CB for 3 h to suppress extrusion of the pseudo-second polar body.

In vitro culture of embryos was performed in 20-μl droplets of Porcine Zygote Medium-5 (PZM-5; Research Institute For the Functional Peptide, Yamagata, Japan) under paraffin oil in a plastic Petri dish maintained in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂ at 38.5 °C. For culturing embryos beyond the morula stage, 10% (v/v) FBS was added to the medium.

Removal of cytoplasmic lipid droplets from the cloned embryos was performed using a noninvasive method reported previously [16], with modifications. Cloned embryos at the morula stage (99–101 h after activation) were treated with 4% (w/v) trypsin (in PBS) for 1 min so that the zonae pellucidae were swollen due to slight digestion. The embryos were then washed twice with PBS + 10% (v/v) calf serum (CS; SAFC Bioscience). The embryos with a swollen zona were centrifuged (12,000 × g, 23 min) at 38 °C in Hepes-TL-PVP containing 7.5 μg/ml CB using a 1.5-ml microcentrifuge tube (Fukae Kasei, Hyogo, Japan) to polarize the cytoplasmic lipid droplets in the perivitelline space. After centrifugation, the embryos were cultured for 1 h until vitrification.

The cryopreservation of embryos was carried out by vitrification using the MVC method [22] as described previously [17]. All solutions used during vitrification and warming were prepared using a basal medium composed of TCM199 (Nissui Pharmaceutical) containing 20 mM Hepes, 4.2 mM NaHCO₃, 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulfate. The embryos were equilibrated with an equilibration solution containing 7.5% (v/v) ethylene glycol (EG; Nacalai Tesque), 7.5% (v/v) DMSO and 20% CS for 4 min, followed by exposure to a vitrification solution containing 15% EG, 15% DMSO, 0.5 M sucrose and 20% CS. The embryos were then loaded onto an MVC plate (Cryotop, Kitazato BioPharma, Shizuoka, Japan) and immediately plunged into liquid nitrogen. The process, beginning with embryo exposure to the vitrification solution and ending with plunging, was completed within 1 min. The embryos were warmed by immersing the MVC plate directly in a warming solution containing 1 M sucrose and 20% CS at 38.5 °C for 1 min. The recovered embryos were transferred to a diluent solution containing 0.5 M sucrose and 20% CS where they were kept for 3 min, after which they were kept for 10 min in a washing solution containing 20% CS in order to remove the cryoprotectant. All solutions, except for the warming solution, were maintained at room temperature.

Crossbred (Large White / Landrace × Duroc) prepubertal gilts weighing 100 to 105 kg were used as recipients of the vitrified cloned embryos. To induce estrus, 1000 IU of eCG (ASKA Pharmaceutical) was injected intramuscularly, followed by an injection of 1500 IU hCG (Kawasaki Pharmaceutical, Kanagawa, Japan) 66 h later. The pigs used in the present study were maintained in a seminidowless facility with a controlled temperature (15–30 °C) and received a standard pig diet twice a day and water ad libitum. All of the animal experiments in the present study were approved by the Institutional Animal Care and Use Committee of Meiji University (IACUC-05-002).

The vitrified cloned embryos were cultured for about 40 h prior to transfer. The embryos that developed to the blastocyst stage were selected for transfer. The zonae pellucidae were removed from the blastocysts by digestion in 0.25% (w/v) pronase (in PBS) for 10 to 60 sec at 38.5 °C. The embryos were transferred to the uterine horns of the recipient gilts approximately 146 h after hCG injection.

Nonvitrified cloned embryos were transferred to sexually matured gilts (Large White / Landrace × Duroc, 130 – 180 kg) after estrus synchronization. The pregnant gilts were aborted by an i.m. injection of 1 mg of a prostaglandin F₂α analog (Cloprostenol, Estrumate; Intervet, Osaka, Japan) between 25 and 40 days after mating, followed by a second injection of 0.5 mg Cloprostenol 24 h later. At the same time as the second Cloprostenol injection, 1000 IU of eCG was administered (i.m.). Ovulation was induced by administering an i.m. injection of 1500 IU hCG approximately 67 h after eCG. The reconstructed embryos were cultured for 24 to 48 h prior to transfer, and then morphologically normal embryos at the 1-2-cell stage on day 1 and those at the 2-8-cell stage on day 2 were selected for transfer to the oviducts of the recipients approximately 51 h after hCG injection.

Data were analyzed using the SPSS 16.0 software (SPSS, Chicago, IL, USA). The differences in the developmental rates of the vitrified and nonvitrified cloned embryos were analyzed by the χ² test. The level of significance was set at P<0.05.

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References


