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Study of Electro-Fusion/Activation in Somatic Cell Nuclear Transfer to Obtain Cloned Putian Black Pig Embryos

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Abstract: Putian Black pigs, an improved pig breed in China, are on the edge of extinction and urgently require protection. To establish a method of electro-fusion/activation for reconstructed embryos obtained by Somatic Cell Nuclear Transfer (SCNT), the applicability of microelectrode fusion in electro-fusion/activation of reconstructed embryos from SCNT and its optimal parameters were explored using pig oocyte maturation *in vitro* for 42-44 h as receptors and ear fibroblast as donors. Researchers also compared microelectrode fusion with traditional chamber fusion and the effects of different culture media (PZM-3 and NCSU-23) on reconstructed embryos were evaluated. Researchers found that the optimal parameters for microelectrode fusion/activation were 16 V, 20 μsec and 1 DC in which the fusion efficiency was significantly higher than that of chamber-fusion (84.8 vs. 64.4%) but the rates of embryo cleavage and blastocyst formation showed no statistical difference ($p>0.05$). The cleavage rates of embryos cultured in PZM-3 or NCSU-23 showed no statistical difference ($p>0.05$) while the rate of blastocyst formation in PZM-3 was higher than that in NCSU-23 (13.8 vs. 7.84%, $p<0.05$). These results show that microelectrode fusion/activation can be used in SCNT to obtain cloned embryos from Putian Black pigs and its fusion efficiency is higher than that of traditional chamber fusion. In addition, reconstructed embryo development is optimal in PZM-3. This study establishes a technical basis for SCNT to obtain cloned embryos from Putian Black pigs. Moreover, it can be applied to related fields such as the production of transgenic cloned embryos from Putian Black pigs.

Key words: Putian Black pig, SCNT, electro-fusion, electro-activation, embryo development

INTRODUCTION

Putian Black pigs are one of the elite cultivars of 36 endemic species in China and possess many attractive features such as early maturation and easy fat accumulation, high fertility, a good growth rate on the roughest of forage as well as delicate and soft meat. This breed is a valuable genetic resource among the excellent breeds in all of the world markets. However, its numbers are dropping sharply at a rate of 300 per annum because of the effect of exotic species over the past decade. If allowed to continue, Putian Black pigs will be close to extinction in 10 years which has aroused the concern of the related national departments that have included Putian Black pigs in a list of 12 local protected livestock species in Fujian, China. As an endangered species, Putian Black pigs are in a poor state of conservation.

Somatic Cell Nuclear Transfer (SCNT) has an important application in the conservation of animal breeds (Holt *et al*., 2004; Andrabi and Maxwell, 2007) and has been successfully performed for some endangered and rare animal species including Enderby Island cattle in New Zealand (Wells *et al*., 1998), Argali in Europe (Loi *et al*., 2001), Desert Argali in America (Williams *et al*., 2006), wild cats in Africa (Gomez *et al*., 2004), Gray wolves in Korea (Oh *et al*., 2008) and the Exorcize Dog (Jang *et al*., 2009). All of these studies indicate that we can adopt SCNT to protect Putian Black pigs.

The fusion and activation of reconstructed embryos is one of the key procedures for reprogramming of donor nuclei and initiating the development of reconstructed embryos (Naruse *et al*., 2007). Electro-fusion, an optimal fusion method in nuclear transfer has been used in SCNT to obtain cloned embryos from a variety of animal species (Polejaeva *et al*., 2000). Chamber fusion (Kubota *et al*., 2000; Cho *et al*., 2002) and microelectrode fusion (Du *et al*., 2006; Shen *et al*., 2006) have been reported. Microelectrode fusion has greatly improved the efficiency

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of fusion and activation of cloned embryos because of the accurate targeting of the somatic cell engraftment to make the interface between the donor cell and recipient oocyte perpendicular to the electrical field between the two microelectrodes for appropriate control of oocyte extrusion.

In vitro culture systems of reconstructed embryo are essential to directly affect the process of embryo development and pregnancy (Fujiwara *et al*., 1993). The report on reconstructed pig embryos cultured *in vitro* was rare and the rates of embryo development to morula and blastocyst stages are very low (Sun *et al*., 2001). Accordingly, as the basis of embryo biotechnology, many areas of *in vitro* embryo culture need to be improved. NCSU-23 medium containing BSA has been used in several trials to successfully obtain cloned pigs and is the most commonly used culture medium for pig embryos (Swain *et al*., 2001; Bormann *et al*., 2002). PZM-3 medium has a limited chemical composition based on the flowing liquid in oviducts and has been recently reported to enable more embryos to grow to blastocytes in combination with amino acids (Petters *et al*., 1990).

In this study, ear fibroblasts from Putian Black pigs were used as donor cells and oocytes as recipients to test different fusion methods (Microelectrode Fusion and Chamber Fusion) and the parameters of fusion/activation including field intensity, pulse time and pulse number, to determine their effects on fusion in pig SCNT. In addition, the effects of two embryo culture media on the development of reconstructed embryos were compared simultaneously. The study aimed to find the optimal fusion/activation protocol and elucidate the ideal *in vitro* culture system for reconstructed pig embryos which lays the foundation for establishing a SCNT technology system for Putian Black pigs.

MATERIALS AND METHODS

Reagents: All reagents used in this study were purchased from Sigma (St. Louis, MO, USA), except for those noted.

Manufacture and connection of the microelectrode: The manufacture and connection of the microelectrode were performed according to a report by Liu *et al*. (2007). Two microelectrodes with diameters of 100 and 15 μm, respectively were prepared from platinum thread with a diameter of 200 μm by a grinding apparatus in the laboratory. Each microelectrode, linked with a stainless steel wire with a diameter of 1 mm was bent into a "Z" shape and separately fixed to the two fixed operating rods of a micromanipulator. Then, the wires were connected to the electrodes of a fusion device via two thin wires. Both electrodes were sterilized by 75% ethanol before use. The two microelectrodes were then adjusted to be parallel to the object stage of the micromanipulator.

Preparation of donor cells: The tissue was obtained from Putian Black pigs at a reservation farm, cut into 3×4 mm^2 pieces by a sterile ear puncher and placed in D-PBS containing penicillin and streptomycin. The tissues were transported on ice to the laboratory as quickly as possible and then washed three times in PBS to remove any blood. Hair was removed from the explants by shearing scissors which were then cut into 1 mm^3 pieces with sterile ophthalmic scissors. The tissue explants were then cultured in DMEM containing 10% Fetal Bonine Serum (FBS) at 38.5°C in a humidified atmosphere with 5% CO_2. The medium was changed every 2 days.

Collection of Cumulus Oocyte Complexes (COCs) and maturation *in vitro*: Pig ovaries collected from a local slaughterhouse in Luoyang were transported to the laboratory within 2 h in physiological saline at 37°C. COCs were retrieved from follicles of 3-6 mm in diameter by aspiration with a 12 g hypodermic needle attached to a 10 mL syringe containing a small amount of preheated D-PBS. The COCs were washed three to five times in D-PBS and those with an integral appearance, evenly granulated cytoplasm and enclosed by at least three layers of compact cumulus cells were selected. After three to five washes with *in vitro* maturation medium (TCM199 supplemented with 10 IU mL^{-1} PMSG, 10 IU mL^{-1} HCG, 3 IU mL^{-1} FSH, 10 ng mL^{-1} EGF and 1% (v/v) ITS) the selected COCs were cultured in maturation medium for 42-44 h at 38.5°C in a humidified atmosphere with 5% CO_2.

Enucleation and injection of oocytes: After maturation, the expanded cumulus cells of COCs were removed by repeated pipetting in 0.2% (w/v) hyaluronidase. Oocytes in metaphase-II with a first polar body, uniform cytoplasm and good shape were selected and then washed five times in droplets of enucleation medium (D-PBS supplemented with 5 μg mL^{-1} CB, 0.01% PVA and 10% FBS). These COCs were enucleated after equilibration for 5 min.

Enucleation and injection were conducted simultaneously for nuclear transfer. A 50 μL droplet of enucleation medium containing 5 μg mL^{-1} Hoechst 33342 and 20-30 oocytes and a 30 μL droplet of injection medium (D-PBS containing 10% FBS and donor cells) was prepared in a 30 mm plastic dish under mineral oil. A total of 20-30 donor cells with a smooth membrane were aspirated into a glass pipette containing enucleation medium and placed into the medium containing the oocytes for enucleation.

Each oocyte was held with a holding micropipette (internal diameter: approximately 15-20 μm; external diameter: approximately 15-20 μm) and the zona pellucid was partially dissected with an enucleation needle with a diameter of approximately 15-20 μm. The first polar body and approximately 10-20% of the surrounding cytoplasm, presumably containing the metaphase-II chromosomes were aspirated through the hole made by the enucleation needle which was guided by fluorescence and were then discharged. The donor cells inside the enucleation needle were injected into the perivitelline space of the enucleated oocytes. The cell-oocyte couplets were squeezed gently by the top of the same needle to allow close contact between donors and recipients.

Electro-fusion and activation of reconstructed embryos methods of fusion and activation: The cell-oocyte couplets were transferred into TCM199 medium (pre-equilibrated for 4 h in a CO_2 incubator) and incubated for 30 min after enucleation followed by five washes in electrical fusion medium (0.3 M mannitol, 50 mM $CaCl_2$, 0.1 M $MgSO_4$, 0.27 M histidine and 0.1% BSA) and were then incubated for a further 3-5 min. Fusion was performed by two methods including Microelectrode Fusion and Traditional Chamber Fusion as described.

Microelectrode fusion: Electrical fusion was conducted under an inverted microscope. One microelectrode (inner diameter: 15 μm) was connected to the right fixed pole of a micromanipulator and the other one (diameter: 100 μm) was connected to the left. The donor cells were placed at 3 o'clock using the right electrode that gently pressed against the zona pellucida attached to the donor cell (Fig. 1a) to make the interface between the cell membrane and ooplasm perpendicular to the line between the two electrodes. Then, fusion and activation were induced by different parameters using an electro cell fusion manipulator.

Chamber fusion: The donor-cytoplasm complexes were placed into a fusion chamber (gap between electrodes: 1 mm) containing fusion medium and the position of the donor cell was adjusted to make the interface between its membrane and the ooplasm perpendicular to the electric field direction (Fig. 1b). Fusion was then induced by a 1 DC pulse of 1.20 kV cm^{-1} for 60 μsec. After incubating in a CO_2 incubator for 1 h, the fusion result was observed under a stereomicroscope and the fusion rate was calculated and recorded.

Experimental design of microelectrode fusion and activation

Experiment 1 (Effect of the electrical field on fusion): Reconstructed embryos were randomly divided into three groups and the fusion of the three groups was manipulated by 1 DC pulse of 15, 25 and 35 V for 20 μsec. The results were evaluated by the fusion, cleavage and blastocyst rates of reconstructed embryos.

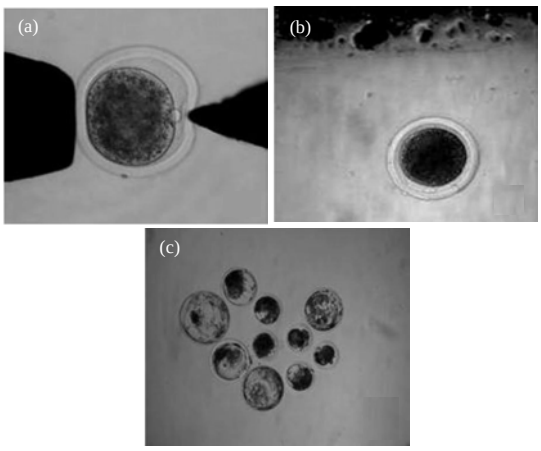

Fig. 1: Methods of electro-fusion and representative photographs of SCNT blastocysts: a) Microelectrode fusion. The original magnification was x200; b) Chamber fusion. The original magnification was x100. c) *In vitro* development of the Putian Black pig embryos reconstructed from somatic cells at day 8 after fusion. The original magnification was x40

Experiment 2 (Effect of the electrical pulse duration on fusion): The optimal electrical field was adopted based on the results of experiment 1. Fusion was induced by 1 DC pulse for 10, 20, 30 and 40 μsec. The results were assessed as described in experiment 1.

Experiment 3 (Effect of the electric pulse frequency on fusion): The optimal electrical field and pulse duration were chosen according to the results of experiments 1 and 2 and the reconstructed embryos were randomly divided into two groups that were exposed to 1 or 2 DC. The results were determined as described in experiment 1.

Experiment 4 (Further exploration of the effect of electrical field on fusion): Based on the results of above experiments, fusion was conducted by the optimal pulse duration and pulse frequency at 12, 14, 16, 18 and 20 V. The results were assessed as described in experiment 1.

Experiment 5 (Effect of microelectrode fusion and traditional chamber fusion on electrical fusion): Microelectrode fusion and traditional chamber fusion were compared to determine their effects on the

fusion/activation and development of cloned embryos based on the results of the above experiments.

***In vitro* culture of cloned embryos:** Reconstructed embryos were cultured in PZM-3 while evaluating the proper fusion condition. After fusion under the optimal condition, the reconstructed embryos were rinsed five times in NCSU-23+0.4% BSA or PZM-3+0.3% BSA and then cultured in 25 μL microdrops of the corresponding embryo medium for at least 2 h in a CO_2 incubator in groups of 10-15 embryos at 38.5°C. Cleavage rates were observed after culture for 48 h. Half of the medium volume was changed after 3 days and the blastocyst rates were recorded at 7-9 days of culture (Fig. 1c).

Statistical analysis: Each experiment was repeated at least three times. The rates of fusion, cleavage and blastocysts of the reconstructed embryos were assessed by the χ^2-test. The difference was considered statistically significant at $p<0.05$.

RESULTS

Experiment 1: This experiment was designed to test the effect of field intensity on electro-fusion. Based on the results of experiment 1 (Table 1), the fusion rate of the 15 V group was 83.3% which was evidently higher than that of the other groups ($p<0.05$). The cleavage rates of the 15 and 25 V groups (78.5 vs. 74.1%) showed no significant difference but were significantly higher than 53.8% of the 35 V group. As, the voltage increased, the blastocyst rate was significantly lower (16.6 vs. 10.2 vs. 0%).

Experiment 2: To elucidate the effect of pulse time on electro-fusion, researchers adopted 10, 20, 30 and 40 μsec. As shown in Table 2, the fusion rate of the 20 μsec group was significantly higher than that of the other groups. Significantly higher rates of cleavage and blastocyst development were obtained at 10, 20 and 30 μsec than those at 40 μsec (74.8, 77.2 and 74.6 vs. 49.2%, respectively for cleavage development and 12.0, 15.8 and 13.6% vs. 4.5%, respectively for blastocyst development).

Experiment 3: To obtain the preferable DC pulse number, the reconstructed embryos were induced by 1 or 2 DC pulses of 15 V for 20 μsec. As shown in Table 3, the rates of fusion and blastocysts resulting from 1 DC pulse were significantly higher than those of 2 DC pulses while both cleavage rates were not significantly different (78.0 vs. 74.6%).

Table 1: Effect of field intensity on the electro-fusion of pig oocytes

Field intensity	No. of couplets	Fusion rate (%)	No. of embryo cleaved (%)	No. of blastocyst (%)
15 V, 20 μsec, 1DC	302	252 (83.3)^a	198 (78.5)^a	42 (16.6)^a
25 V, 20 μsec, 1DC	323	220 (68.1)^b	163 (74.1)^a	22 (10.2)^b
35 V, 20 μsec, 1DC	290	47 (16.2)^c	25 (53.8)^b	0 (0.0)^c

Table 2: Effect of pulse time on the electro-fusion of pig oocytes

Pulse duration	No. of couplets	Fusion rate (%)	No. of embryo cleaved (%)	No. of blastocyst (%)
15 V, 10 μsec, 1DC	302	223 (73.8)^{ab}	167 (74.8)^a	20 (12.0)^b
15 V, 20 μsec, 1DC	288	242 (84.1)^a	187 (77.2)^a	38 (15.8)^a
15 V, 30 μsec, 1DC	310	215 (69.5)^b	160 (74.6)^a	29 (13.6)^a
15 V, 40 μsec, 1DC	298	198 (66.3)^b	97 (49.2)^b	9 (4.5)^b

Table 3: Effect of pulse number on the electro-fusion of pig oocytes

Pulse number	No. of couplets	Fusion rate (%)	No. of embryo cleaved (%)	No. of blastocyst (%)
15 V, 20 μsec, 1DC	304	247 (81.2)^a	193 (78.0)^a	40 (16.4)^a
15 V, 20 μsec, 2DC	296	184 (62.1)^b	137 (74.6)^a	16 (8.9)^b

Table 4: Effect of field intensity on the electro-fusion of pig oocytes

Field intensity	No. of couplets	Fusion rate (%)	No. of embryo cleaved (%)	No. of blastocyst (%)
12 V, 20 μsec, 1DC	294	228 (77.4)^a	161 (70.8)^{ab}	27 (12.0)^a
14 V, 20 μsec, 1DC	286	225 (78.5)^a	165 (73.4)^{ab}	32 (14.2)^a
16 V, 20 μsec, 1DC	300	251 (83.8)^{ab}	197 (78.4)^a	43 (17.1)^a
18 V, 20 μsec, 1DC	310	268 (86.3)^b	200 (74.7)^{ab}	45 (16.8)^a
20 V, 20 μsec, 1DC	308	230 (74.7)^a	159 (69.1)^b	18 (11.3)^a

Table 5: Effect of methods on the electro-fusion of pig oocytes

Fusion methods	No. of couplets	Fusion rate (%)	No. of embryo cleaved (%)	No. of blastocyst (%)
Micro-electrode fusion (16 V, 20 μsec, 1DC)	294	249 (84.8)^a	194 (77.8)^a	42 (16.8)^a
Chamber fusion (120 V, 20 μsec, 1DC)	306	197 (64.4)^b	150 (76.3)^a	28 (14.4)^a

Within the same column, values with different superscripted letters (a-d) are significantly different

Experiment 4: To further test the effect of electric field strength on the fusion of reconstructed embryos, researchers observed the fusion results at 12-18 V. As shown in Table 4, the fusion rate increased with the electric field strength at 12-18 V. The fusion rates at 16 and 18 V which had the maximum fusion rate (86.3%) were significantly higher than the other two groups. However, the voltage did not significantly affect the rates of cleavage and blastocysts whereas the 16 V group had the highest rates of cleavage and blastocyst development. When the voltage was increased to 20 V, the fusion, cleavage and blastocyst rates of reconstructed embryos were significantly lower than those at voltages lower than 20 V.

Experiment 5: The comparison between microelectrode fusion and chamber fusion revealed that microelectrode fusion had a significantly higher fusion percentage (Table 5) while the cleavage and blastocyst rates following fusion/activation did not statistically differ between the two methods ($p>0.05$).

Table 6: Effect of different culture media on reconstructed embryos

Culture media	No. of embryo reconstructed	No. of embryo cleaved (%)	No. of blastocyst (%)
NCSU-23+0.4%BSA	255	141 (55.2)[a]	20 (7.84)[a]
PZM-3+0.3%BSA	246	138 (56.1)[a]	34 (13.8)[b]

Experiment 6: To screen the medium for reconstructed embryos after fusion/activation, the embryos were cultured at 38.5°C in a humidified atmosphere with 5% CO_2 in microdrops of NCSU-23 or PZM-3 media. As shown in Table 6, the two cleavage rates were similar (55.2 vs. 56.1%) but the blastocyst rate in the PZM-3 group was significantly higher than that of the NCSU-23 group (13.8 vs. 7.84%).

DISCUSSION

Effects of different electro-fusion/activation conditions on the reconstructed embryos: Electro-Fusion is a method to merge two or more cells into one in an electric field *in vitro*. The principle is that the electrophoretic power forces the cells in the fusion solution to arrange in pairs or a string of cells randomly under the alternating electrical field. Then, the plasma membranes are disrupted by the instantaneous electric field formed by the high-voltage pulse as a result of producing many reversible holes through the contacting cells in which intracellular materials are exchanged followed by membrane fusion (Zimmermann, 1982).

For cell electro-fusion, a prerequisite is establishment of close contact between the cell membranes. Second, the interface must be perpendicular to the electric field direction (Zimmermann and Vienken, 1982). The situation in which adjacent cells do not contact closely or fail to contact results in a low rate of fusion or even non-fusion (Miyoshi *et al*., 2001). SCNT often results in donor cells separating from the perivitelline space of the ooplasm or sticking to the zona pellucida which causes difficulty for fusion of the donor-cytoplast pairs without other external factors.

The activation efficiency of oocytes by the electrical pulse is directly related to the electric field strength and pulse duration. In this study, SCNT was performed by electric fusion and activation simultaneously. The present study used microelectrode fusion/activation and investigated the effect of electric field strength, pulse duration and pulse numbers on the rates of fusion, cleavage and blastocysts. Researchers found that the rates of fusion, cleavage and blastocysts were optimal when the parameters were 16 V, 20 μsec and 1 DC.

In most SCNT studies, chamber fusion is the typical method for electro-fusion which is conducted in a small chamber between a pair of parallel electrodes (Meng *et al*., 1997). However, for cloned embryos obtained by SCNT, it is difficult to place the contacting surface of the transplanted somatic cells and oocytes exactly perpendicular to the electric field direction between the two poles, resulting in unsuccessful cell fusion. Furthermore, the condition in which cloned embryos are entirely inside the electrical field causes serious cytoplasmic damage that affects the efficiency of fusion/activation of cloned embryos and further development.

Recently, some researchers have begun to use a pair of microelectrodes to study fusion/activation. However, the top of the microelectrodes are all round (the top should be a flat surface) with a diameter much longer than that of the donor cells (Takeuchi *et al*., 1999). Wilmut *et al*. (1997) reported that the fusion efficiency using a microelectrode is better than that of chamber fusion. Du *et al*. (2006) used granulosa cells and skin cells as donor cells in microelectrode fusion and obtained fusion rates of 59±5.6 and 33±3.9%, respectively. Kishi *et al*. (2000) reported the fusion rate in SCNT of bovine fibroblasts and mammary epithelial cells as 5±4 and 56±7%, respectively. Shen *et al*. (2006) used goat skin fibroblasts as donor nuclei and the fusion rate after SCNT was 64.8%. The compactness of the interface is one of the key factors that affect the fusion efficiency. In the study, researchers used microelectrodes that can not only accurately make the interface between the donor and receptor vertical to the electric field direction but also squeeze the donor cell sticking to the zona pellucida or contacting with the ooplasm loosely to the surface of the ooplasm. The compactness of the contacting surface is increased and the membrane connection between the cell and ooplasm is established in this manner. As a result, the fusion efficiency is apparently improved. The study showed that the fusion rate of microelectrode fusion was significantly higher than that of chamber fusion (84.8 vs. 64.4%). However, both of the methods did not show a statistical difference in the cleavage and blastocyst rates of cloned embryos.

The study elucidated that microelectrode fusion significantly increases the fusion efficiency of reconstructed embryos compared with that of the traditional chamber fusion. Moreover, many fusion/activation experiments indicated that the optimal parameters of microelectrode fusion are 16 V, 20 μsec and 1 DC.

Effects of different media on the *in vitro* development of fused reconstructed embryos: In recent years, the systems for *in vitro* embryo culture have become the basis of the relevant studies including in-depth studies

of SCNT and directly affect the success of research. The medium used for embryo culture is one of the important factors that may cause a low developmental rate. NCSU-23 is one of the successful mediums among the *in vitro* culture mediums for pig embryos. However, PZM media (PZM-3 and PZM-4) are newly developed media that very closely resemble the porcine physiological environment *in vivo* (Yoshioka *et al*., 2002). Im *et al*. (2006) reported that they eventually obtained cloned pigs after transplantation of cloned embryos cultured in PZM-3.

There are two main differences between NCSU-23 and PZM-3. First, PZM-3 contains essential and non-essential amino acids without taurine whereas NCSU-23 contains taurine but not essential or non-essential amino acids. Second, the energy substrates of PZM-3 are calcium lactate and sodium pyruvate. In NCSU-3, glucose is the energy substrate that supports embryo development. Amino acids are important nutrients for embryo development and play multiple roles at different stages of embryo development (Steeves and Gardner, 1999) such as synthesis of proteins, nucleotides and signaling molecules, providing nutrition and energy, adjusting osmotic pressure and pH and preventing oxidation. Kikuchi *et al*. (2002) discovered that the composition of the medium is extremely important for pig embryos at 2-3 days of development when embryo arrest is closely related to the composition of the medium. Their study proposed that it is conducive for pig embryos *in vitro* to exceed the 4 cell stage using pyruvate and lactate rather than glucose because glucose in NCSU-23 might be detrimental to the early development of embryos.

Researchers compared the effects of two conventional embryo culture media on the development of cloned embryos. The results showed that the cleavage rate of cloned embryos cultured in PZM-3 was not significantly different from that of cloned embryos cultured in NCSU-23 but the blastocyst rate in PZM-3 was significantly higher than that in NCSU-23 (13.8 vs. 7.84%). However, a report by Yoshioka *et al*. (2002) greatly differs from the conclusion; this difference might result from the level of oocyte maturation and the different conditions of the laboratories.

CONCLUSION

The present study established a technical basis for SCNT to obtain cloned embryos from Putian Black pigs and suggested that researchers can adopt SCNT to protect Putian Black pigs.

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