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# Development before and after cryopreservation of porcine parthenogenetic embryos derived from early-delipated oocytes

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## Research Article

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**ABSTRACT**

The present study investigated the effect of delipation (lipid droplet removal) on the developmental competence of porcine oocytes. Delipated (+/−) and/or vitrified (+/−) oocytes were subjected to parthenogenetic activation, the viability of the early parthenogenetic embryos was recorded, and the embryos were then transferred to recipients for further development. The results showed that the cleavage and blastocyst rates of the parthenogenetic embryos from the early delipated oocytes were significantly lower than those of embryos from a control group (undelipated oocytes) ($P<0.05$). After transfer to recipient pigs, these embryos were able to further develop and to produce parthenogenetic fetuses. Using the minimum volume cooling (MVC) method, the parthenogenetic embryos from early delipated oocytes could be cryopreserved by vitrification at 1-cell to early blastocyst stages. After thawing, the early blastocyst stage was found to be optimal for vitrification. Also embryos vitrified at 2-4 cells derived from early delipated oocytes were transferred into two recipient pigs, and resulted in limb-bud stage fetuses. In conclusion, the results demonstrated that parthenogenetic fetuses can be produced from porcine embryos before or after vitrification by a strategic combination of *in vitro*-matured (IVM) oocyte delipation with vitrification at any early embryonic developmental stage. This approach may have application to cryopreservation of cloned and intracytoplasmic sperm injection (ICSI) embryos derived from delipated oocytes.

## INTRODUCTION

The cryopreservation of mammalian oocytes and embryos has application to preserve genetic diversity and for *in vitro* fertilization protocols in humans. To date, cryopreservation techniques have been used in a variety of species, including rabbits [1, 2], cattle [3, 4], horses [5, 6], cats [7, 8], mice [9, 10] and ferrets [11], among others. However, because of the biological uniqueness of porcine oocytes, the survival and blastocyst formation after verification was still lower [12].

The failure to vitrify porcine oocytes and embryos successfully is thought to be due predominantly to high intracellular lipid content of oocytes [13] and the wide cell volume [14, 15]. By removing the cytoplasmic lipid droplets (delipation), the sensitivity of porcine oocytes and early-stage embryos to low temperature is remarkably reduced. Nagashima et al. [16] and Li et al. [17] showed that live, healthy offspring were obtained from delipated, *in vitro*-fertilized and nuclear transfer embryos following cryopreservation. Delipated, *in vitro*-matured (IVM) porcine oocytes could be subjected to freezing/warming and then developed to the blastocyst stage after parthenogenesis [18] and *in vitro* fertilization [19] or into fetuses after parthenogenesis [20].

Based on the above studies, the cryopreservation of porcine delipated embryos would be of greater practical value than that of oocytes. Nevertheless, during porcine somatic cell nuclear transfer (SCNT) and intracytoplasmic sperm injection (ICSI), the damage to the reconstructed embryos is exacerbated because of twice handling (micromanipulation and delipation), which not only directly influences embryos developmental stability and reduces embryos survival rate, but also increases the difficulty of operation. Delipation of IVM oocytes combined with SCNT and ICSI can reduce labor and avoid second handling. In addition, the developmental characteristics of parthenogenetic embryos resemble those of *in vitro* fertilized embryos [21], so parthenogenetic embryos can be considered as an ideal model system to analyze the developmental competence of cryopreserved embryos derived from delipated porcine IVM oocytes.

The present study was undertaken to investigate the influence of porcine oocyte delipation on developmental competence following parthenogenesis with and without vitrification. Furthermore, we determined the developmental ability of vitrified-warmed, early parthenogenetic embryos during various developmental stages.

## MATERIALS AND METHODS

All animal experiments were performed with the approval of the Animal Care Committee of Yunnan Agricultural University.

### Chemicals

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

### *In Vitro Maturation of Oocytes*

After obtaining permission to use animal parts in this study, porcine ovaries were collected from the Hongteng slaughterhouse (Chenggong Ruide Food Co., Ltd., Kunming, Yunnan Province, China) and transported to the laboratory in 0.9% (w/v) NaCl solution at 25-30°C. The oocytes were obtained and cultured using the same method previously described [22]. In brief, cumulus-oocyte complexes were obtained from follicles 3 mm to 6 mm in diameter and were selected with at least three layers of compacted cumulus cells. Approximately 50 oocytes were cultured in 200 ml drops of maturation medium at 38.5°C in an atmosphere with 5% $CO_2$ (100% humidity) (APC-30D, ASTEC, Japan).

### *Removal of Cytoplasmic Lipid Droplets from the Oocytes (Delipation)*

The removal of cytoplasmic lipid droplets from the oocytes was performed as previously described [16]. The denuded oocytes were incubated in porcine zygote medium-3 (PZM-3) supplemented with 5.0 μg/ml CB (cytochalasin B) for either 1.5 or 2.5 h and then centrifuged in the same medium in 1.5 ml microcentrifuge tubes at 13,200 × g for 25 min at room temperature. The resultant lipid layer was removed from the oocytes **(Figure 1)** by micromanipulation using a beveled suction pipette (outside diameter, 20-25 μm) attached to a micromanipulator under an inverted microscope (IX71, Olympus, Tokyo, Japan). After lipid removal, the oocytes were washed and maintained in PZM-3 at 38.5°C.

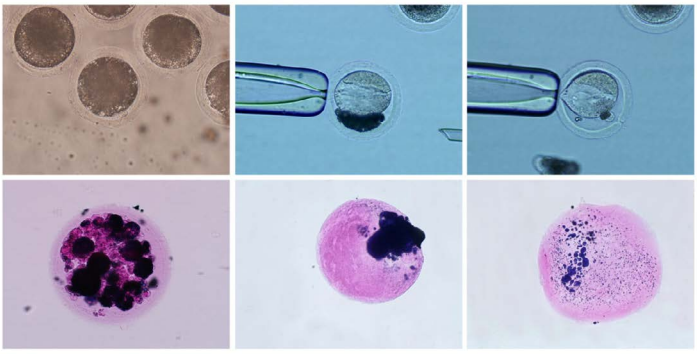

**Figure 1.** Photomicrographs showing the morphology of porcine *in vitro*-matured oocytes before and after delipation. Oocytes under the light microscope (a), enlarged to show staining with Sudan black B (b), and after centrifugation to separate the lipid layer (c). The cytoplasmic lipid droplets stained with Sudan black B was shown after centrifugation before their aspiration (d). The morphology of oocytes was shown after delipation (e and f), demonstrating reduced staining with Sudan black B. The arrows indicate the lipid droplets.

### *Parthenogenetic Activation of Oocytes and In Vitro Development*

Groups of 10-20 early delipated oocytes and undelipated oocytes (controls) were washed twice in activation solution consisting of 0.3 M mannitol, 0.5 mM HEPES, 0.01% BSA (fatty acid free), 0.01 mM $CaCl_2$, and 0.01 mM $MgCl_2$. Then, the oocytes were placed in a chamber connected to an electro cell fusion generator (LF201, Nepa Gene Co., Ltd., Japan) with two parallel electrodes (1.0 mm apart) overlaid with activation solution. A single direct current pulse was applied to activate the oocytes at output voltages of 150 V/mm for 1 × 100 μsec. After activation, the oocytes were incubated in PZM-3 supplemented with 2.2 μg/ml CB for 2 h and then cultured in 25 μl droplets of PZM-3 medium without CB in humidified air containing 5% $CO_2$ at 38.5°C. The normal development of the oocytes to different stages was recorded at 1, 2, 3, 5 and 7 days of culture, respectively. The cell number of the blastocysts was counted after fixing and Hoechst 33342 staining under an ultraviolet light microscope.

***Lipid droplet stain***

The cytoplasmic lipid droplets of oocytes and parthenogenetic embryos were stained with Sudan black B, a lipophilic dye. Briefly, the oocytes or embryos were fixed in a 10% formaldehyde solution for 3 min, washed in distilled water, dried and then stained in drops of 1% Sudan black B (w/v) in 70% ethanol for 4 min, followed by rinsing in 70% ethanol and drying. The material was then placed in 1% eosin for 3 min washed in distilled water, and dried. The prepared oocytes or embryos were finally mounted in gelatin on cover slips and examined under a light microscope at 400X magnification.

***Vitrification of Embryos***

The cryopreservation of embryos was performed by vitrification using the MVC method as described previously [23]. All of the solutions used during vitrification and warming were prepared using a basic medium composed of TCM-199 (M5017) containing 20 mM HEPES, 4.2 mM $NaHCO_3$, 75 µg/ml potassium penicillin G and 50 µg/ml streptomycin sulfate. The embryos derived from the early delipated oocytes were washed 3 times in HEPES-buffered Tyrode's medium (TLH) containing 0.1% (w/v) polyvinylalcohol (PVA) (TLH-PVA), equilibrated with an equilibration solution containing 7.5% (v/v) ethylene glycol, 7.5% (v/v) DMSO and 20% FBS for 4 min followed by exposure to a vitrification solution containing 15% EG, 15% DMSO, 0.5 M sucrose and 20% FBS within 30 sec. The embryos were then loaded onto a MVC plate and immediately plunged into liquid nitrogen. This process was completed within 1 min. After 1~3 days, the embryos were thawed by immersing the MVC plate directly into a thawing solution containing 1 M sucrose and 20% FBS at 38.5°C for 1 min. The recovered embryos were transferred to a diluent solution containing 0.5 M sucrose and 20% FBS for 3 min and then subjected to two 5-min exposures to a washing solution containing 20% FBS to remove the cryoprotectants. All of the solutions were maintained at room temperature, except the warming solution.

***Embryo transfer***

Crossbred (Large White/Landrace Duroc) gilts were used as surrogate mothers for the 2- to 4-cell-stage parthenogenetic embryos, as previously described [22]. At 0 and 9 h after the first standing estrus was exhibited, reconstructed embryos cultured for 6 and 30 h after activation were surgically transferred to the oviducts of the estrous surrogate mother through the fimbriae. Pregnancy was detected approximately day 23 after surgical transfer using an ultrasound scanner (HS-101V, Honda Electronics Co., Ltd., Yamazuka, Japan).

***Experimental design***

**Experiment1. Development of parthenogenetic embryos derived from early delipated oocytes**

Oocytes extruded the first polar body were selected for the removal of their cytoplasmic lipid droplets and pretreated with 5 µg/ml CB for either 1.5 h or 2.5 h. Each treated group contained 25-30 oocytes. After the parthenogenetic activation of the early delipated oocytes, the developmental ability of embryos, including their cleavage and blastocyst rates, were examined and compared. To investigate the *in vivo* developmental competence of parthenogenetic embryos, excellent embryos were surgically transferred to the oviducts of the surrogate mothers. Pregnancy in the recipients was detected by ultrasound scanning, and the parthenogenetic fetuses were surgically recovered at day 26 or 27 of gestation.

**Experiment2. Development of parthenogenetic embryos derived from early delipated oocytes after vitrification-thawing**

Parthenogenetic embryos from Experiment 1 with high cleavage and blastocyst rates were directly cryopreserved using the MVC method at the 1-cell, 2- to 4-cell, 5- to 8-cell, morula and early blastocyst stages. The morphology **(Figure 2 e and f)** and further developmental ability of the embryos after thawing was examined. The 2- to 4-cell-stage embryos were transferred after thawing to recipient gilts. Pregnancy in the recipients was identified by ultrasound scanning at approximately day 23, and the parthenogenetic fetuses were surgically recovered.

***Statistical analysis***

Analysis of variance (ANOVA, PROC GLM) was performed using the SAS statistical package (SAS Institute Inc., Cary, NC, USA). $P<0.05$ was considered to be statistically significant.

## RESULTS

**Development of parthenogenetic embryos derived from early delipated oocytes**

To evaluate the effect of cytoplasmic lipid droplets removal on the developmental competence of oocytes, delipated oocytes were subjected to parthenogenetic activation, and then the cleavage and blastocyst rates were recorded. The embryos derived from oocytes that were and were not delipated could develop normally to the cleavage stage, but the cleavage rates of the 1.5 and 2.5 h CB-treated groups were significantly lower than those of the control group (54.2% and 64.6% compared with 82.8% respectively, $P<0.05$; **Table 1, Figure. 2a and c**). After culture for 7 d *in vitro*, the blastocyst rates were 20.2% and 32.6% for the 1.5 h and 2.5 h treatments with CB after delipation, respectively, and 41.6% for the control, indicating significant differences among the three groups ($P<0.05$; **Table 1, Figure 2 b and d).** The cell number of blastocysts did not differ significantly among the three groups ($P>0.05$).

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**Table 1.** The developmental ability of parthenogenetic embryos from delipated oocytes treated with CB

| Treatment | No. of embryos (replications) | No. of cleavage (%) | No. of blastocysts (%) | Cell number of blastocysts |
|---|---|---|---|---|
| 1.5 h | 431 (5) | 239 (54.2 ± 7.5)[a] | 92 (20.0 ± 6.5)[a] | 46.9 ± 10.4 |
| 2.5 h | 880 (10) | 569 (64.6 ± 7.9)[a] | 272 (32.6 ± 9.7)[b] | 42.0 ± 5.7 |
| Control* | 1025 (10) | 845 (82.8 ± 7.7)[b] | 429 (41.6 ± 8.1)[c] | 38.1 ± 8.7 |

Values with different superscript letters within a column are significantly different ($P<0.05$).
*Embryos derived from intact oocytes.

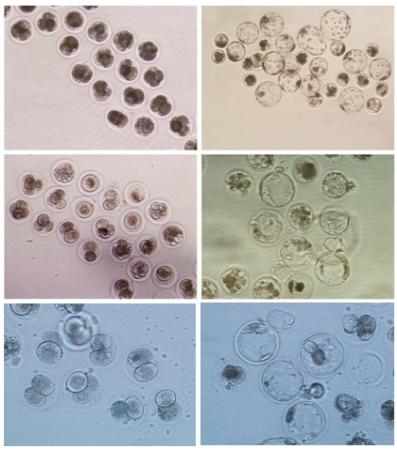

**Figure 2.** Morphological comparisons of parthenogenetic embryos derived from oocytes that were and were not delipated. The morphology of embryos derived from undelipated oocytes was shown at the cleavage (a) and blastocyst (b) stages. Following oocyte delipation, the embryonic morphology was maintained at the cleavage (c) and blastocyst (d) stages. The embryos derived from delipated oocytes were shown at the cleavage (e) and blastocyst (f) stages after thawing.

The differences in embryos derived from oocytes that were and were not delipated were obvious in photomicrographs taken after staining with Sudan black B. The lipid droplets appeared as black or blue-black pellets, allowing the 2-cell and morula embryos from undelipated oocytes to stain deeply. After delipation, most of the black or blue-black pellets (i.e., the lipid droplets) were removed from the oocytes, and the 2-cell and morula embryos appeared pink **(Figure 3).**

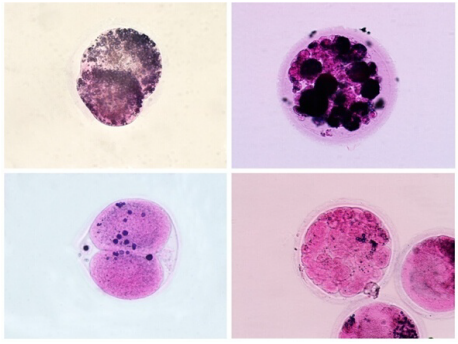

**Figure 3.** Photomicrograph of porcine parthenogenetic embryos derived from delipated and undelipated oocytes by *in vitro* maturation. The morphology of an embryo from an undelipated oocyte was shown at the 2-cell (a) and morula (b) stages. After oocyte delipation, the embryonic morphology was maintained at the 2-cell (c) and morula (d) stages.

To investigate the *in vivo* developmental competence of parthenogenetic embryos, excellent embryos from early delipated oocytes were surgically transferred to the oviducts of two surrogate mothers whose pregnancies were detected by ultrasound scanning. Either 23 or 41 parthenogenetic fetuses were recovered from the two surrogate mothers by laparotomy at day 26 or 27 of gestation, including 4 or 5 live, 9 or 16 dead and 10 or 20 absorbed fetuses, respectively **(Table 2).** The average weights of the 4 live and 9 dead fetuses recovered at day 26 were 0.3967 g and 0.1732 g, respectively. The average weights at day 27 of the 5 live and 16 dead fetuses were 0.5163 g and 0.3772 g, respectively **(Table 3 and Figure 4 b).**

**Table 2.** Development of parthenogenetic embryos from delipated oocytes after transfer to recipient gilts.

| Recipient | No. of transferred embryos | Pregnancy | Gestation days | No. of developing embryos | | | |
|---|---|---|---|---|---|---|---|
| | | | | Total | Live | Dead | Absorbed |
| 1 | 260 | + | 26 | 23 | 4 | 9 | 10 |
| 2 | 152 | + | 27 | 41 | 5 | 16 | 20 |

A plus sign (+) indicates pregnancy.

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**Table 3.** The weights of the live and dead fetuses obtained at 26 and 27 days of gestation.

| Embryo | Weight (g) on different days | |
|---|---|---|
| | 26 | 27 |
| Live | 0.3967 ± 0.0493 | 0.5163 ± 0.0826 |
| Dead | 0.1732 ± 0.0853 | 0.3772 ± 0.1030 |

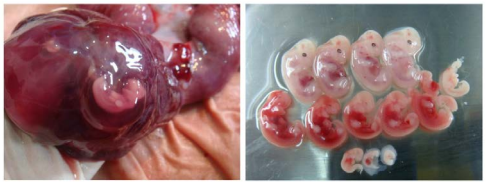

**Figure 4.** Developmental ability of porcine parthenogenetic embryos derived from early delipated oocytes. A dead fetus at day 29 developed from an embryo frozen-thawed at the 2- to 4-cell stage (a). Day 26 fetuses developed from embryos frozen-thawed at the 2- to 4-cell stage (b). The live fetuses are positioned in the top row; the dead fetuses are positioned in the two bottom rows.

### Development of parthenogenetic embryos derived from early delipated oocytes after vitrification and thawing

Based on Experiment 1, the parthenogenetic embryos derived from delipated oocytes were cryopreserved at the 1-cell, 2- to 4-cell, 5- to 8-cell, morula and early blastocyst stage using the MVC method to examine the viability of vitrified, thawed embryos. The result showed that vitrified, thawed embryos could develop to the cleavage and blastocyst stages **(Figure 2 e and f).** Among the above groups, the blastocyst rate was highest in the embryos vitrified during the early blastocyst stage. Although there was no statistically significant difference in the blastocyst rate among the groups (P>0.05), the 2- to 4-cell-stage embryos tended to show a higher blastocyst rate (19.3%) than did of the 1-cell- and 5- to 8-cell-stage embryos (14.6% and 14.7%, **Table 4**).

**Table 4.** The *in vitro* developmental ability of vitrified embryos derived from delipated oocytes after thawing at various stages

| Embryo | 1-Cell | 2- to 4-Cell | 5- to 8-Cell | Morula | Early blastocyst |
|---|---|---|---|---|---|
| No. vitrified and thawed (replications) | 234 (9) | 362 (10) | 218 (8) | 98 (7) | 28 (3) |
| No. developing (%) | 108 (45.4 ± 18.8) | 149 (41.2 ± 12.7) | 108 (49.5 ± 9.0) | - | - |
| No. of blastocysts (%) | 35 (14.6 ± 5.0) | 70 (19.3 ± 5.8) | 32 (14.7 ± 2.2) | 22 (22.4 ± 6.3) | 16 (57.1 ± 8.4) |

To evaluate the *in vivo* developmental competence of 2- to 4-cell-stage embryos after thawing, we transferred embryos to two surrogate mothers. One recipient was identified as pregnant through ultrasound scanning. Laparotomy at day 29 of gestation detected 19 fetuses, of which 10 were dead and 9 were absorbed **(Table 5 and Figure 4 a).**

**Table 5.** Development after derivation from delipated oocytes of parthenogenetic embryos following vitrification and transfer to recipient gilts.

| Recipient | No. of transferred embryos | Pregnancy | Gestation days | No. of developing embryos | | | |
|---|---|---|---|---|---|---|---|
| | | | | Total | Live | Dead | Absorbed |
| 1 | 287 | + | 29 | 19 | 0 | 10 | 9 |
| 2 | 132 | – | – | 0 | 0 | 0 | 0 |

A plus sign (+) indicates pregnancy; a minus sign (–)indicates no pregnancy.

## DISCUSSION

Porcine embryos are intrinsically sensitive to low temperatures because of the large number of intracytoplasmic lipid droplets present in their oocytes. Thus, the embryos produced *in vitro* [24], *in vivo* [25], by cloning [26] or by oocyte fertilization [16] has a low survival rate or capacity for subsequent development after cryopreservation. It has been suggested that the cryotolerance of embryos would be increased after pretreatments such as delipation [27], cytoskeletal stabilization [28] or membrane composition alteration [29].

The present study has evaluated the developmental competence of parthenogenetic embryos derived from delipated porcine IVM oocytes. Because the developmental characteristics of parthenogenetic embryos resemble those of *in vitro* fertilized embryos [21], parthenogenesis can provide a valuable measure of oocyte competence and be considered as an ideal model system. Our results showed that vitrified, parthenogenetic embryos derived from delipated porcine IVM oocytes could develop to the fetal stage. This result could be used to porcine SCNT and ICSI. During SCNT and ICSI, the zona pellucida of an oocyte is destroyed to inject somatic cells or sperm into the oocyte. Delipation during an early stage of SCNT and ICSI embryos increased the difficulty of operation and damage to the embryos. By contrast, the delipation of oocytes during the injection of somatic cells or sperm avoided a second handling of the embryos, which saved time and decreased the embryonic damage.

In this study, we found that before delipation, treatment with CB could greatly reduce the operation time and increase the survival rate of early delipated oocytes (data not shown). CB is a microfilament inhibitor that can disrupt actin polymerization and

prevent cytokinesis without affecting karyokinesis. So treatment of cells with CB makes the plasma membrane less rigid and more elastic so that microfilaments are disrupted during micromanipulation [30]. In addition, our results showed that the developmental ability of 2.5 h treatments with CB was higher than that of 1.5 h treatment group. It is likely that treatment with CB for 2.5 h has a positive effect and no obvious toxicity, on the contrary, the long interference of cytoplasmic trafficking resulting from actin–tubulin depolymerization negatively affects development when treatment with CB for 3-5 h [31].

In our study, the developmental competence of parthenogenetic embryos derived from delipation oocytes did not improve compared with control group. This result was similar to those of previous studies [18,19], which showed that the blastocyst formation rate of IVF and parthenogenetic embryos derived from delipated IVM oocytes was low. The cytoplasmic lipid droplets of oocytes were able to specifically provide energy until placental development in pigs and play a role in the synthesis of second messengers during embryonic development [32]. So removal of lipid droplets would be expected to have negative effects on the development of oocytes. Although the roles of lipid droplets in the cytoplasm were not clarified in this study, the porcine parthenogenetic embryos derived from delipated IVM oocytes could be successfully developed to the blastocyst stage *in vitro* and to the limb-bud stage *in vivo*. Thus, the role of lipid droplets in porcine oocytes should be further investigated.

In addition, our results showed that the optimal time for vitrification was the early blastocyst stage, the blastocyst rate of re-expansion was 57.1% after thawing, a rate as high as that of vitrified, parthenogenetic embryos [23, 33]. The vitrified, delipated porcine embryos at the morula and expanded blastocyst stages were able to achieve a high postwarming survival rate, in accordance with previous reports [23, 34, 35,36]. This result may have occurred because the reduced lipid content coincided with an increased tolerance to freezing in the late-stage embryos [37].

Regarding stage specific differences in tolerance to freezing, the developmental ability of parthenogenetic embryos vitrified at various cleavage stages were compared in the present study. Although no significant difference was observed in statistics, the 2- to 4-cell stage embryos showed a tendency of higher blastocyst rate than those of 1-cell and 5- to 8-cell stages embryos. Moreover, the pregnancy rate is very low when the blastocyst was transferred to surrogate. So we chose vitrified-warmed 2- to 4-cell stage embryos to transfer to surrogate mothers and were able to develop to the limb-bud stage *in vivo*. Note that compared with delipation during the initial embryonic developmental stage, the earlier delipation of oocytes reduced the damage inflicted on the zona pellucida caused by removing the polarized lipid droplets. The successful cryopreservation of porcine parthenogenetic embryos derived from early delipated oocytes can facilitate not only the long-term preservation of valuable genes but also research on these genes and on parthenogenesis.

In conclusion, the present study demonstrated that porcine parthenogenetic embryos from early delipated oocytes were able to develop to blastocysts *in vitro* and to the limb-bud stage *in vivo*. Furthermore, the delipated oocytes could be vitrified at any early embryonic stage and continued to develop after thawing. This approach has great potential because it demonstrated that porcine SCNT and ICSI embryos derived from delipated oocytes could be directly cryopreserved, a discovery that can promote the application of SCNT and ICSI technologies.

## ACKNOWLEDGEMENT

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