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# Developmental Ability of Porcine *In Vitro* Matured Oocytes at the Meiosis II Stage After Vitrification

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**Abstract.** The aim of the present study was to investigate whether a combination of cytoplasmic lipid removal (delipation) and treatment by a microtubule stabilizer, paclitaxel, would lead to efficient cryopreservation of porcine *in vitro* matured (IVM) oocytes at the meiosis II (MII) stage. Vitrification and subsequent re-warming and culture of 109 untreated oocytes produced only 9 blastocysts (8.3%). On the other hand, the post-vitrification blastocyst rate was significantly improved (21/113, 18.6%, P<0.05) when oocytes were treated with 1 $\mu$M paclitaxel. Oocyte delipation also significantly increased the post-vitrification blastocyst rate compared with the untreated group (15/37, 40.5%, P<0.05). The delipation-and-paclitaxel group exhibited a significantly higher blastocyst rate (34/75, 45.3%, P<0.05) than the paclitaxel group, although it was not significantly higher than that for the delipation group. In transfer experiment, a total of 109 (18.6%) parthenogenetic blastocysts were obtained from 586 oocytes vitrified with the delipation-and-paclitaxel treatment. Transfer of 72 blastocysts to two recipients resulted in 14 (19.4%) somite stage fetuses. In conclusion, we demonstrated for the first time that by removing cytoplasmic lipid droplets from oocytes and performing a microtubule stabilization procedure, vitrified porcine IVM MII-stage oocytes could efficiently develop to the blastocyst stage while retaining the ability to develop into fetuses.

**Key words:** Cryopreservation, Delipation, *In vitro* matured oocytes, Pig, Vitrification

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Cryopreservation techniques for mammalian oocytes and embryos have been used for three main purposes: to preserve the genes of elite livestock animals and increase the efficiency of animal breeding, preserve valuable genetically modified animals and endangered species and use germ cells effectively in assisted reproductive technology. Thus far, embryo cryopreservation as a practical technique has been used in a variety of species, including experimental and livestock animals [1, 2]. On the other hand, cryopreservation of oocytes remains impractical compared with embryos, and successful production of progeny from cryopreserved oocytes has been reported in limited species, including rabbits [3], cattle [4], horses [5], cats [6], humans [7], mice [8] and rats [9]. As with porcine embryos, unfertilized porcine oocytes are highly sensitive to low temperature [10]. Consequently, cryopreserved porcine oocytes have yet to be used to successfully produce viable piglets.

One of the reasons for the low-temperature sensitivity of porcine oocytes and embryos is their high intracellular lipid content. Nagashima and colleagues showed that the freezing tolerance of porcine oocytes [11] and early-stage embryos [12, 13] can be dramatically increased by removing their cytoplasmic lipid droplets using a process called delipation.

It is known that the meiotic spindle of the oocyte is extremely cryosensitive, and the impaired development of oocytes at meiosis II after cryopreservation has been attributed to damage to the spindle [14]. Paclitaxel is known to stabilize the microtubules that constitute the spindle [15]. It has been reported that the normality of the spindles of MII-stage oocytes immediately after freezing can be improved by paclitaxel treatment in both pigs [16] and cattle [17]. Furthermore, the developmental potency of cryopreserved MII-stage oocytes has been shown to be improved by paclitaxel treatment in mice [18], cattle [19], sheep [20] and pigs [16].

The aim of the present study was to investigate whether a combination of delipation and paclitaxel treatment would lead to efficient cryopreservation of porcine MII-stage oocytes. Here, we are the first to demonstrate that porcine MII-stage oocytes can be cryopreserved while retaining their ability to develop into fetuses.

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## Materials and Methods

### *Animal care*

The pigs used in the present study were maintained in a semi-windowless facility with controlled temperature (15–30 C) and received a standard pig diet twice a day and water ad libitum. All of the animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Meiji University (IACUC 05-002).

### *Chemicals*

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

*In vitro maturation of oocytes*

Porcine 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 $\mu$g/ml potassium penicillin G, 50 $\mu$g/ml streptomycin sulfate, 2.5 $\mu$g/ml amphotericin B and 0.1% (w/v) polyvinyl alcohol (PVA). Cumulus-oocyte complexes were collected from the ovarian antral follicles (3.0 to 6.0 mm in diameter) by aspirating with a 10-ml syringe and a 20-gauge hypodermic needle, and those with at least three layers of compacted cumulus cells were selected and cultured in maturation medium (NCSU23 medium [21] supplemented with 0.6 mM cysteine, 10 ng/ml epidermal growth factor, 10% (v/v) porcine follicular fluid, 75 $\mu$g/ml potassium penicillin G and 50 $\mu$g/ml streptomycin sulfate). Maturation culture was performed in the presence of 10 IU/ml eCG (ASKA Pharmaceutical, Tokyo, Japan) and 10 IU/ml hCG (ASKA Pharmaceutical) at 38.5 C in a humidified atmosphere of 5% $CO_2$ in air for 22 h. The oocytes were then cultured for an additional 18 h without eCG or hCG under a gas atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ [22]. *In vitro* matured oocytes with expanded cumulus cells were treated with 1 mg/ml of hyaluronidase dissolved in Tyrode lactose medium containing 10 mM Hepes and 0.3% (w/v) polyvinylpyrrolidone (Hepes-TL-PVP) and separated from the cumulus cells by gentle pipetting. Oocytes with an evenly granulated ooplasm and an extruded first polar body were selected for the subsequent experiments.

*Removal of cytoplasmic lipid droplets from oocytes (Delipation)*

IVM oocytes were treated with 4% (w/v) trypsin (in PBS) for 2–3 min so that the zonae pellucidae were swollen due to slight digestion. The oocytes were then washed twice with PBS + 10% (v/v) calf serum (CS; SAFC Biosciences, Kansas City, KS, USA) to eliminate the effect of trypsin. Oocytes with swollen zona were then cultured in maturation medium containing 7.5-$\mu$g/ml cytochalasin B (CB) for 30 min.

To remove lipid droplets from ooplasm, the oocytes were centrifuged at 38 C in Hepes-TL-PVP containing 7.5-$\mu$g/ml CB using a 1.5-ml microcentrifuge tube (Fukae Kasei, Hyogo, Japan). For *in vitro* survival assessment of the vitrified oocytes, the centrifuge conditions were set at 12,000 × *g* for 23 min according to our previously reported procedure [11, 23]. In transfer experiments for the vitrified oocytes, the centrifuge conditions were reduced to 9,000 × *g* for 5 min to minimize possible damage to the oocytes. After centrifugation, the oocytes were checked to determine whether they had polarized lipid droplets in the perivitelline space.

*Paclitaxel treatment*

Delipated oocytes as well as non-delipated oocytes were kept in maturation medium for 60 min prior to vitrification. Some of the delipated and non-delipated oocytes were incubated in the presence of 1 $\mu$M paclitaxel during the last 30 min. When these oocytes were vitrified, 1 $\mu$M paclitaxel was added to the equilibration and vitrification solutions.

*Vitrification of MII stage oocytes*

Cryopreservation of oocytes was carried out by vitrification using the minimum volume cooling (MVC) method [24, 25]. All solutions used during vitrification and re-warming were prepared with a basal medium composed of TCM199 (Nissui Pharmaceutical) containing 20 mM Hepes, 4.2 mM $NaHCO_3$, 75 $\mu$g/ml potassium penicillin G, and 50 $\mu$g/ml streptomycin sulfate. Oocytes were equilibrated stepwise with 7.5% (v/v) and 15% ethylene glycol (EG; Nacalai Tesque, Kyoto, Japan) for 30 sec, respectively. Equilibrated oocytes were transferred to vitrification solution containing 30% EG and 0.5 M sucrose (Nacalai Tesque) as cryoprotective agents (CPA), loaded onto an MVC plate (Cryotop; Kitazato BioPharma, Shizuoka, Japan) and then immediately plunged into liquid nitrogen. This process, beginning with oocyte exposure to a vitrification solution and ending with plunging into liquid nitrogen, was completed within 1 min. After storage for 1 to 125 days, oocytes were re-warmed by immersing the MVC plate directly into a re-warming solution containing 1 M sucrose for 1 min. Dilution of CPA was carried out in a two-step manner; that is, the oocytes were placed in dilution solutions containing 0.5 M and 0.25 M sucrose for 2 min, respectively, after which they were kept for 10 min in a washing solution to remove the CPA. All solutions contained 20% (v/v) CS and were maintained at 39 C.

*Induction of parthenogenetic development of the vitrified oocytes*

Vitrified-rewarmed oocytes and intact control oocytes were cultured in NCSU23 medium supplement with 4 mg/ml bovine serum albumin (Fraction V) for 1 to 1.5 h and then subjected to electrical activation. The oocytes were washed twice in an activation solution composed of 280 mM mannitol (Nacalai Tesque), 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.01% (w/v) PVA and aligned between two wire electrodes (1.0 mm apart) in a drop of the activation solution placed on a fusion chamber slide. A single direct current pulse of 150 V/mm was applied for 100 $\mu$sec using an electrical pulsing machine (LF201; NEPA GENE, Chiba, Japan). Activated oocytes were treated with 5 $\mu$g/ml CB for 3 h to suppress extrusion of the second polar body.

*In vitro culture of embryos*

*In vitro* culture of the activated oocytes was performed in 20-$\mu$l droplets of Porcine Zygote Medium-5 (PZM-5; Research Institute for the Functional Peptides, Yamagata, Japan) under paraffin oil in a plastic Petri dish, which was maintained in a humidified atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ at 38.5 C. For culture of parthenogenic embryos beyond the morula stage, 10% (v/v) fetal calf serum (SAFC Bioscience) was added to the medium.

*Fixation and staining of embryos*

Blastocysts developed from activated oocytes were fixed with aceto-alcohol (1:3) 7 days after activation. Blastocysts were then stained with 1% (w/v) aceto-orcein to determine the number of cells.

*Transfer of vitrified oocytes*

Crossbred (Large White/Landrace × Duroc) prepubertal gilts weighing from 100 to 105 kg were used as recipients of the embryos. For induction of estrus, gilts were intramuscularly injected with 1000 IU of eCG (ASKA Pharmaceutical) followed by

**Table 1.** *In vitro* development of porcine IVM oocytes at the MII stage after vitrification

| | Delipation | Paclitaxel | Vitrification | No. of oocytes | | | Mean no. of cells ± SEM |
|---|---|---|---|---|---|---|---|
| | | | | Cultured | Normally cleaved (%) | Developed to the blastocyst stage (%) | |
| Untreated | – | – | + | 109 | 37 (33.9) [a] | 9 (8.3) [a] | 50.3 ± 10.3 [a] |
| Paclitaxel | – | + | + | 113 | 45 (39.8) [a] | 21 (18.6) [b] | 56.7 ± 8.0 [a] |
| Delipation | + | – | + | 37 | 23 (62.2) [ab] | 15 (40.5) [bc] | 64.0 ± 9.6 [a] |
| Delipation-and-paclitaxel | + | + | + | 75 | 41 (54.7) [ab] | 34 (45.3) [c] | 56.5 ± 4.9 [a] |
| Intact control | – | – | – | 104 | 77 (74.0) [b] | 76 (73.1) [d] | 97.2 ± 4.9 [b] |

[a, b, c, d] Values with different superscripts differ significantly (P<0.05).

1500 IU of hCG (Kawasaki Pharmaceutical, Kanagawa, Japan) 66 h later. Vitrified oocytes were cultured after activation for 6 days, and those that developed to blastocysts with a normal appearance were selected for transfer. The selected blastocysts were treated with 0.25% (w/v) pronase (in PBS) for 20 sec at 38.5 C to remove the zona pellucida. Blastocysts with the zona removed were transferred to the uterine horns of recipient gilts at 148–150 h after hCG injection. The recipients were autopsied on day 23 of gestation to collect fetuses. The fetuses obtained were fixed with 4% (w/v) paraformaldehyde, embedded in paraffin blocks and stained with Hematoxylin-Eosin after thin-sectioning. The Morphology was then compared between these fetuses and fetuses derived from non-vitrified parthenogenetic embryos that had been obtained in our previous study (unpublished).

*Statistics*

The data were analyzed using the SPSS 16.0 software (SPSS, Chicago, IL, USA). Proportional data were subjected to arcsine transformation and evaluated by one-way analysis of variance (ANOVA) followed by multiple comparisons using a Tukey test. Differences in the number of cells per blastocyst between groups were also determined using one-way ANOVA followed by multiple comparisons using a Tukey test. The level of significance was set at P<0.05.

## Results

*In vitro development of vitrified porcine MII oocytes*

MII-stage oocytes were divided into the following five groups: untreated group, paclitaxel group, delipation group, delipation-and-paclitaxel and intact control group (Table 1). Survival of the vitrified oocytes could not be judged by their morphological appearance immediately after re-warming; hence, all of the recovered oocytes were cultured to assess *in vitro* development. Vitrification and subsequent re-warming and culture of 109 untreated MII-stage oocytes (untreated group) produced only 9 blastocysts (8.3%). On the other hand, the post-vitrification blastocyst rate was significantly improved (21/113, 18.6%, P<0.05) when MII-stage oocytes were treated with 1 $\mu$M paclitaxel (paclitaxel group). Oocyte delipation (delipation group) significantly increased the post-vitrification blastocyst rate compared with the untreated group (15/37, 40.5%, P<0.05). The delipation-and-paclitaxel group exhibited a significantly higher blastocyst rate (34/75, 45.3%, P<0.05) than the paclitaxel group, although it was not significantly higher than that for the delipation group. The blastocyst formation rate and number of cells per blastocyst of the delipation-and-paclitaxel group (34/75, 45.3%; 56.5 ± 4.9) were significantly lower than those of the intact control oocytes (76/104, 73.1%; 97.2 ± 4.9, P<0.05). The pre- and post-vitrification morphologies of the oocytes in the four experimental groups as well as their *in vitro* development are shown in Fig. 1.

*In vivo development of vitrified porcine MII oocytes*

Parthenogenetic development was induced in 586 vitrified MII-stage oocytes, and 109 oocytes (18.6%) developed to blastocysts. Of these, 72 blastocysts were transferred to two recipient pigs. As a result, 14 (19.4%) fetuses, including four exhibiting growth retardation, were produced. The success rates of transferred embryos developing into fetuses in the two pigs were 10/35 (28.6%) and 4/37 (10.8%).

Fig. 2A shows a day 23 fetus developed from the vitrified oocytes, exhibiting the same developmental stages and similar development of the lung buds, atria, ventricles, liver and pancreas as a fetus derived from non-vitrified oocytes (Fig. 2B).

## Discussion

An unfertilized mammalian oocyte is significantly larger than the blastomere of an early embryo or a somatic cell and thus exhibits a small surface area relative to volume. Dehydration of unfertilized oocytes and the penetration of cryoprotectants is therefore highly complex and difficult to achieve. These are factors commonly thought to be responsible for the difficulty in cryopreserving unfertilized oocytes [14].

It has been reported that in cryopreserved MII-stage oocytes, damage occurs in the meiotic spindle, actin filaments and microtubules [26]. Abnormalities of the spindle in particular are serious problems that relate directly to loss of fertilization and developmental abilities [14]. The microtubule, one of the components of the spindle, is a cylindrical structure made of 13 protofilaments bundled together, while a protofilament is a heterodimer consisting of $\alpha$-tubulin and $\beta$-tubulin. It is believed that paclitaxel stabilizes microtubules by initially strengthening the bond between $\alpha$-tubulin and $\beta$-tubulin and then strengthening the link between microtubules by influencing microtubule-associated proteins, in addition to other mechanisms [15]. A previous study also demonstrated that pacli-

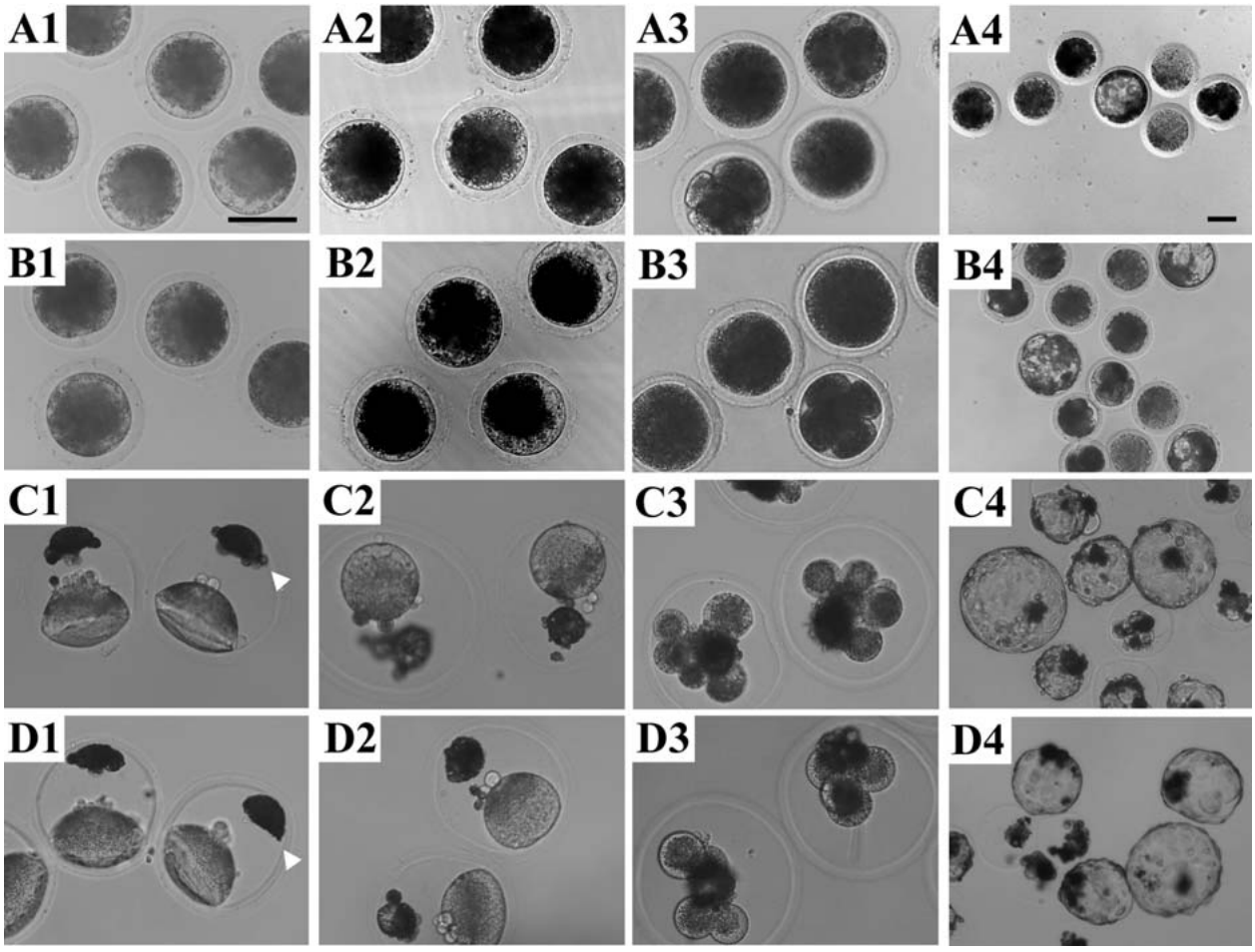


**Fig. 1.** Morphological appearance and *in vitro* development of porcine IVM oocytes vitrified with different pretreatments. Oocytes vitrified without pretreatments (A1–A4), with paclitaxel treatment (B1–B4), with delipation (C1–C4) and with delipation-and-paclitaxel treatment (D1–D4). A1, B1, C1, D1: Oocytes before vitrification. Cytoplasmic lipid droplets polarized in the swollen zona pellucida by delipation treatment are shown by arrowheads (C1, D1). A2, B2, C2, D2: Oocytes recovered after vitrification. Note that lipid droplets remained separated from the ooplasm in the delipated oocytes after vitrification (C2, D2). A3, B3, C3, D3: Vitrified oocytes at the 4- to 8-cell stage 2 days after activation. A4, B4, C4, D4: Blastocysts developed from the vitrified oocytes 7 days after activation. A1–A3, B1–B3, C1–C3 and D1–D3 (× 200) and A4, B4, C4 and D4 (× 100) were obtained at the same magnification. Scale bar=100 $\mu$m.

taxel treatment improved the normality of mitochondria distribution after cryopreservation in porcine MII-stage oocytes [27]. This may have occurred because the distribution of mitochondria in the oocytes was maintained by microtubules.

The present study also confirmed that the *in vitro* post-cryopreservation developmental ability of porcine MII-stage oocytes was improved by paclitaxel treatment. However, using the post-vitrification developmental rate as a criterion, it appears that the viability of the oocytes was improved by delipation and that paclitaxel treatment played only a supplemental role. Further investigation is required to determine whether paclitaxel treatment is essential in preserving the ability of porcine oocytes to develop into fetuses during vitrification.

The non-invasive delipation method used in this study allows simultaneous removal of cytoplasmic lipid droplets from many oocytes at one time. This confers a significant advantage compared to existing methods in which micromanipulation is performed on each oocyte [12, 13, 23]. However, non-invasive delipation requires a procedure for expanding the zona pellucida using proteolytic enzymes. This is a very delicate procedure that dissolves the zona pellucida gently using trypsin. Our success rate using this method was 88% (data not shown). Excessive or insufficient digestion of the zona pellucida results in damage during centrifugation or incomplete removal of lipid droplets. These two factors represent the key disadvantages of non-invasive delipation.

In the present study, the zona pellucida was removed before transfer of the blastocysts derived from vitrified oocytes. This procedure was performed based on the findings of our preliminary experiments showing that both vitrified oocytes and non-vitrified oocytes treated with non-invasive delipation cannot hatch follow-

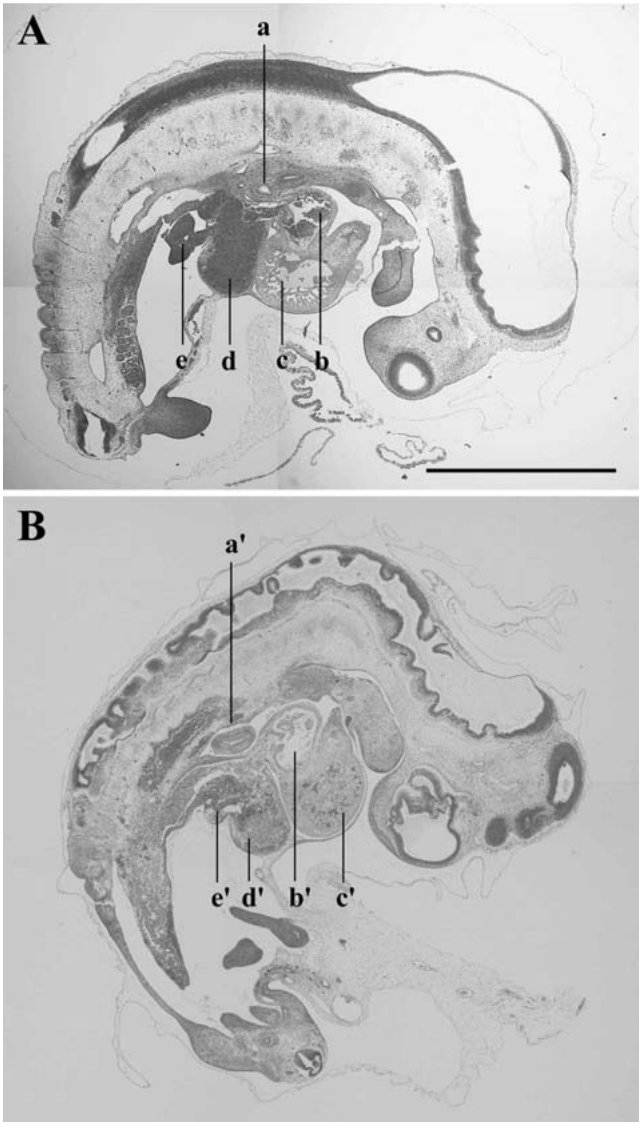


**Fig. 2.** Somite stage (day 23) fetuses developed from vitrified and non-vitrified oocytes. The top image (A) shows a Hematoxylin-Eosin stained section of a fetus developed from the vitrified oocytes exhibiting similar development of the lung buds (a, a'), atria (b, b'), ventricles (c, c'), liver (d, d') and pancreas (e, e') as those of a fetus (B) derived from non-vitrified oocytes. Scale bar=2.0 mm.

ing *in vitro* development to blastocysts (unpublished data). Furthermore, we were unable to achieve successful conception in an experiment in which blastocysts derived from non-invasively delipated morula were transferred without removal of the zona pellucida (unpublished data). Since the zona pellucida of the porcine blastocyst disappears *in utero* before implantation [28], non-invasively delipated oocytes may have trouble undergoing the mechanisms associated with the intrauterine disappearance of the zona pellucida. Further investigation is required to investigate the effect of non-invasive delipation on the *in vitro* hatching of porcine embryos and the *in vivo* disappearance of the zona pellucida.

Thus far, several reports have investigated cryopreservation of porcine MII-stage oocytes [29–31], all of which have confirmed oocyte development to the blastocyst stage, but none of which successfully produced piglets. Du *et al*. [32] improved the cleavage and blastocyst formation rates of vitrified porcine MII-stage oocytes using high hydrostatic pressure before vitrification. On the other hand, our experiments revealed a very high blastocyst rate (45.3%) using a combination of paclitaxel treatment and non-invasive delipation as pretreatments for cryopreservation of porcine MII-stage oocytes, and further confirmed development of oocytes to the fetal stage in embryo transfer experiments. To our knowledge, the present study is the first to report the successful development of cryopreserved porcine unfertilized oocytes into fetuses. The fetuses derived from the vitrified oocytes were shown to exhibit the same developmental stage and anatomical features as fetuses derived from non-vitrified oocytes, and we considered the effect of vitrification on the fetal development to be minimal.

The present study showed that vitrified porcine MII-stage oocytes had the ability to develop to somite stages of development. However, vitrified oocytes need to be fertilized to show whether they have the ability to develop into normal piglets. *In vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are among the options required to prove this. IVF requires determining conditions, e.g., appropriate sperm concentration and insemination hours, for MII-stage oocytes possessing a zona pellucida that has been somewhat digested by trypsin. As with ICSI, it may be significantly difficult to hold an oocyte with a swollen zona pellucida at the proper position using a holding pipette. Furthermore, because centrifugation leads to movement or collapse of the first polar body, it is difficult to inject sperm into an oocyte while avoiding MII-stage chromosomes using the polar body as a landmark. Wu *et al*. [33] successfully obtained piglets by performing IVF with porcine MII-stage oocytes whose zonae pellucidae had been removed. This method may be an option for fertilizing vitrified MII-stage oocytes.

In conclusion, we have shown that by removing cytoplasmic lipid droplets from oocytes and performing a microtubule stabilization procedure, vitrified porcine IVM MII-stage oocytes could efficiently develop to the blastocyst stage while retaining the ability to develop into fetuses.

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