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Birth of piglets from *in vitro*-produced, zona-intact porcine embryos vitrified in a closed system

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Abstract

As the importance of swine models in biomedical research increases, it is essential to develop low-cost, high-throughput systems to cryopreserve swine germplasm for maintenance of these models. However, porcine embryos are exceedingly sensitive to low temperature and successful cryopreservation is generally limited to the use of vitrification in open systems that allow direct contact of the embryos with liquid nitrogen (LN₂). This creates a high risk of pathogen transmission. Therefore, cryopreservation of porcine embryos in a “closed” system is of very high importance. In this study, *in vitro*-produced (IVP) porcine embryos were used to investigate cryosurvival and developmental potential of embryos cryopreserved in a closed system. Optimal centrifugal forces to completely disassociate intracellular lipids from blastomeres were investigated using Day-4 embryos. Cryosurvival of delipidated embryos was investigated by vitrifying the embryos immediately after centrifugation, or after development to blastocysts. In this study, centrifugation for 30 min at 13,000 g was adequate to completely delipidate the embryos; furthermore, these embryos were able to survive cryopreservation at a rate comparable to those centrifuged for only 12 min. When delipidated embryos were vitrified at the blastocyst stage, there was no difference in survival between embryos vitrified using OPS and 0.25 mL straws. Some embryos vitrified by each method developed to term. These experiments demonstrated that porcine embryos can be cryopreserved in a closed system after externalizing their intracellular lipids. This has important implications for banking swine models of human health and disease.

Keywords

Porcine embryos; Delipidation; Vitrification; Closed system

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1. Introduction

Germplasm cryopreservation offers a cost-effective alternative to maintain live animal models in biomedical research. This is especially important with the explosive increase of genetically modified animal models in recent years [1]. Sperm and embryo cryopreservation are routine procedures for banking of mouse and rat models. However, similar protocols for banking swine germplasm are currently inefficient, largely due to difficulties associated with cryopreservation of porcine sperm and embryos. Since the use of pigs in biomedical research has dramatically increased for use as disease models and potential organ donors in xenotransplantation [2,3], there is an increased need for effective methods to maintain swine models in the form of cryopreserved embryos. Cryopreservation of genetically-modified swine models safe-guards unique genotypes from several potential problems, including: (a) genetic drift, (b) genetic instability, (c) genetic contamination, and (d) loss due to disease or catastrophic disasters to housing facilities. Importantly, it also allows models to be maintained only as “cryopreserved lines”, thereby reducing live animal housing requirements and costs. Embryo cryopreservation also facilitates global exchange of swine genetics, due to its cost effectiveness, as well as aiding in management of potential disease transmission and other health concerns.

Maintenance and storage of animal models in the form of cryopreserved germplasm requires that the cells are stored in a closed system, which can keep germplasm in a pathogen-free environment during cryopreservation and storage to protect it from potential contamination with pathogens from various sources, such as LN₂ [4-6]. The commonly used 0.25 mL mini-straws that can be sealed at both ends will serve this purpose. However, due to the exceeding sensitivity of porcine embryos to low temperatures, cryopreservation in conventional closed 0.25 mL mini-straws without any pretreatment is very difficult [7,8].

The sensitivity of porcine embryos to low temperature is mainly due to their high levels of intracellular lipids [9,10]. Removal of intracellular lipids through micromanipulation after lipid polarization by high speed centrifugation of the embryos allows porcine embryos to avoid chilling injury. The first live births from cryopreserved porcine embryos resulted from this approach [10]. Currently, this method is most effective for the cryopreservation of porcine embryos derived both *in vivo* and *in vitro* [10-13].

A second approach to reduce the chilling sensitivity of porcine embryos is based on the knowledge that chilling injury can be minimized by dramatically increasing the cooling rate so that embryos quickly pass through the temperature-sensitive zone during cooling [14,15]. Achieving high cooling rates, such as those used with vitrification, require one to minimize the volume (and therefore the thermal mass, of the medium), as well as to maximize the surface area to volume ratio of the sample. To accomplish this, special devices, such as open pulled straws (OPS), superfine OPS, cryotop, microdroplet method, etc, have typically been used [12,16-27].

Unfortunately, the two approaches described above either damage the zona pellucidae of delipidated embryos or allow direct contact of the sample with LN₂, respectively, thus increasing the chances of pathogen transmission during embryo cryopreservation and storage [4-6]. Therefore, a protocol that allows the porcine embryos to be cryopreserved with an intact zona pellucida in a closed system is needed.

To retain intact zona pellucidae, a non-invasive version of the mechanical delipidation approach was developed and successfully used for generation of live piglets from cryopreserved porcine embryos [16,18-20]. However, complete delipidation of porcine oocytes is beneficial to maximize their cryotolerance [18,28,29], especially when a closed system is used (e.g. 0.25 mL mini straws) [18]. Non-invasive, complete separation of

intracellular droplets from blastomeres was achieved by partial digestion of the zona pellucidae [30], or by using a hyperosmotic solution. Live births were derived from *in vitro*-generated embryos, using the latter approach [13]. However, both approaches used ultrarapid cooling rates which required direct contact between LN₂ and the embryo-containing solution.

In the present study, a series of experiments were conducted to test the hypotheses that: (1) complete separation of intracellular lipids from blastomeres of early stage embryos can be achieving using high-speed centrifugation for extended intervals; (2) cryosurvival of embryos delipidated using this approach is comparable to those delipidated using commonly used centrifugation time; (3) embryos delipidated by this approach could be successfully vitrified in both an open system (*e.g.* OPSs) and a closed system (*e.g.* 0.25 mL mini-straws); and (4) the embryos pretreated by this approach have the potential to develop full term *in vivo* after cryopreserved in either OPS or 0.25 mL mini-straws. We used IVP porcine embryos to test these hypotheses.

2. Materials and methods

2.1. Oocyte maturation, fertilization and culture *in vitro*

Unless specifically stated, all chemicals were obtained from Sigma Aldrich Co (St. Louis, MO, USA). Immature porcine oocytes were obtained from a commercial source (ART Inc, Madison, WI, USA). The cumulus-oocyte complexes (COCs) were delivered overnight in groups of 50 oocytes/tube in plastic tubes containing 2 mL of TCM199-based Phase I maturation medium [31] in a commercial shipper capable of maintaining the temperature at 38.5 °C. Upon reception, tubes containing COCs were transferred into an incubator without opening the tubes. Phase II maturation plates were made by placing 500 µL gonadotropin (FSH and LH)-free phase II medium Phase, provided by the company, into Nunc 4-well plates covered with mineral oil and placed into an incubator for equilibration. After 22 h in Phase I medium, maturing COCs, in a group of 50, were transferred into phase II medium in Nunc 4-well plates, and then cultured for an additional 20-22 h. After maturation, COCs were freed of cumulus cells by 3 min of vortexing. Groups of 30 matured oocytes were then inseminated with frozen-thawed semen in a modified Tris-buffered medium (mTBM) as previously described [32]. Briefly, frozen boar semen (100 µL pellet) was thawed in 10 mL of Dulbecco's modified PBS (D-PBS) and washed twice in D-PBS by centrifuging at 1,900 g for 4 min each. After washing, sperm were re-suspended in mTBM and oocytes were inseminated in mTBM at a final concentration of 5×10^5 sperm/mL in 100 µL mTBM drops. After 6 h of sperm-oocyte co-culture, presumptive zygotes were cultured in a group of 30 in 500 µL PZM-3 with 3 mg/mL fatty acid-free BSA [33]. The incubator used for oocyte maturation, fertilization, and culture was maintained at 38.5 °C with 5% CO₂ in air and maximal humidity. Embryos reached 4-8 cell stages on Day 4 (Day of IVF was considered Day 0) and blastocysts at approximately 144 h post-insemination.

2.2. Externalization of intracellular lipids

Porcine embryos at the 4-8 cell stage (Day 4 post insemination) were selected and sorted into two groups: 4- and 6-8 cell stage embryos. Each group of embryos was placed in a 1.5 mL Eppendorf centrifuge tube containing 1 mL TL-Hepes with 7.5 µg/mL cytochalasin B. The embryos were then subjected to centrifugation at various centrifugal forces for 12 or 30 min depending on the experiments.

2.3. Thermodynamic properties of the vitrification solution

The vitrification solution used in this experiment consisted of TCM199-Hepes + 20% FBS + 20% ethylene glycol (EG) + 20% dimethyl sulfoxide (DMSO) + 0.5 M sucrose, as

previously reported [34]. To ensure that this solution would achieve vitrification, we characterized the thermodynamic properties of this solution using differential scanning calorimetry (DSC), as previously described [35]. Ten 10 μ L of vitrification solution were sealed in a 10 μ L aluminum crucible. Each sample was cooled to -160 °C from 1 °C (held for 1 min) at 100 °C/min in a Pyris Diamond DSC (Perkin-Elmer Corp., Norwalk, CT, USA) using LN₂ as the cooling fluid. After being held at -160 °C for 1 minute, the sample was heated to 20 °C at a rate of 10 °C/min. Distilled water was used as reference standard under the same DSC conditions. Thermograms were recorded for both cases. The thermal lag was corrected based on the water data using a modified Illers' equation [36]. The glass transition (vitrification) temperature (T_g) was calculated using a "half C_p extrapolated" method [37]. Both T_g and the devitrification temperature (T_d) were obtained.

2.4. Embryo vitrification and *in vitro* viability assay

After centrifugation, embryos were either subjected to vitrification immediately (Experiment 2), or after additional culture until reaching the blastocyst stage (Experiment 3). The open pulled straws used were made by pulling 0.25 mL mini-straws softened over a heated plate and had an inner diameter of 0.8 mm, approximately half of the original inner diameter of the 0.25 mL mini-straws, as described previously [34]. For OPS vitrification, embryos in groups of five were first exposed to TCM199-Hepes + 20% FBS with 10% EG and 10% DMSO for 3 min [26,34] and then transferred into the vitrification solution. Embryos were loaded into OPS within 1 min. Warming was conducted by direct immersion of the OPS tip containing the embryos into 0.5 M sucrose in Hepes-buffered TCM199 supplemented with 20% FBS. After 5 min in the 0.5 M sucrose solution, embryos were subsequently exposed to TCM199-Hepes with 20% FBS + 0.25 M sucrose (5 min), TCM199-Hepes + 20% FBS (5 min), and then washed in TCM199-Hepes + 20% FBS.

For vitrification in closed 0.25 mL straws, porcine embryos were pretreated as described in OPS vitrification. Embryos in groups of 10 were loaded into a 0.25 mL straw containing three parts of vitrification solution (separated by small air bubbles) with the embryos in the middle part. After the straw was sealed by heat, vitrification was achieved by immersing the first two parts of vitrification solution in the straw into LN₂ until it solidified, followed by the whole straw. Warming was achieved by warming the straw in air for 8-10 s, and then in 37 °C water for 15-20 s. The contents of the straw were then released into a 35 mm Petri dish containing 2 mL of 0.5 M sucrose in TCM199-Hepes supplemented with 20% FBS. After 5 min in 0.5 M sucrose, the embryos were subjected to serial rehydration as described in OPS vitrification.

To assess the survival of vitrified-warmed embryos *in vitro*, groups of 30 embryos were cultured in 500 ml PZM-3 supplemented with 10% FBS in Nunc 4-well plates covered with mineral oil in an incubator maintained at 38.5 °C, 5% CO₂ in air with maximal humidity. For vitrified-warmed Day 4 embryos, survival was assessed by the formation of blastocysts after 48 h of culture. For vitrified-warmed Day 5 blastocysts, survival was assessed based on the level of recovery into their original spherical shape. Those recovered into their original shape with a fully re-expanded blastocoel (full recovery) and those that only partially regained their spherical shape (partial recovery) were regarded as viable blastocysts.

2.5. Embryo transfer (ET)

After warming, IVP porcine embryos were treated with 0.1% pronase in Dulbecco's modified phosphate buffered saline to remove the zona pellucidae. Embryos were then loaded into a Tom Cat Catheter in a group of 50-55, and embryo transfer was conducted through insertion of the catheter into the oviduct through the infundibulum *via* a midventral incision. Embryos were then deposited into the oviduct near the uterine horn of

synchronized surrogates. All surgical procedures were approved by the Animal Care and Use Committee of the University of Missouri-Columbia.

2.6. Experiment design

Experiment 1 was designed to test the hypothesis that complete separation of intracellular lipids from porcine embryos can be achieved by centrifuging embryos at 4-8 cell stages for extended periods of time at high centrifugal forces. To do this, *in vitro*-produced porcine embryos on Day 4 were subjected to a 2×2 factorial design with two-levels of embryo stage (4-vs 6-8 cell stages) and two levels of centrifugal forces (13,000 g vs 16,000 g). Day 4 embryos at the 4-8 cell stage were selected and sorted into two groups, 4-cell stage embryos or 6-8 cell stage embryos. Those embryos were then subjected to centrifugal forces at 13,000 g and 16,000 g for 30 min to determine the optimal centrifugal force for the complete separation of intracellular lipids from the embryos. After centrifugation, the degree of lipid separation was examined under a stereomicroscope (Olympus America Inc, Center Valley, PA, USA). Embryos were classified into two groups based on the degree of lipid droplet separation from the blastomeres: those with completely disassociated lipid droplets, and those with lipid droplets still associated with blastomeres through string-like structures [30]. An optimized centrifugation protocol was determined. After centrifugation, embryos were subjected to *in vitro* culture to assess the ability of externalized lipid droplets to migrate back into the embryonic cells.

Experiment 2 was designed to test the hypotheses that (1) the optimized centrifugal protocol, as determined in Experiment 1 (one that completely externalizes the intracellular lipids), will not compromise the *in vitro* developmental potential of porcine embryos compared to those treated with shorter centrifugation time after vitrification [18-20]; and (2) removal of zona pellucida will increase the developmental potential of vitrified/warmed embryos. To test these hypotheses, we vitrified 4-8 cell stage embryos using OPS. Embryos at the 4-8 cell stages were processed at the optimal centrifugal force and time and then were subjected to vitrification with OPS. After being stored in LN₂ for 24 h, embryos were warmed, rehydrated and cultured to the blastocyst stage for assessment of their developmental potential after cryopreservation. Cryopreservation results in zona hardening in porcine embryos [13,38]; therefore, a group of vitrified embryos were treated with 0.1% pronase to remove the zona pellucidae to evaluate the effect of zona removal on the developmental potential of vitrified/warmed embryos. Controls included those centrifuged for 12 min and cryopreserved (control 1), as well as non-centrifuged and non-vitrified (control 2).

Experiment 3 was designed to test the hypothesis that completely delipidated porcine blastocysts can be cryopreserved in a closed system using 0.25 mL French mini-straws. Porcine embryos were delipidated on Day 4 and then returned to culture until Day 5. On Day 5, blastocysts with visible externalized lipid droplets were selected [13] and then subjected to vitrification using either OPS or 0.25 mL mini-straws. Vitrified embryos were stored in LN₂ for 24 h. After warming, cryosurvival was assessed by determining the number of blastocysts that were able to regain a spherical shape and re-establish a blastocoel after 24 h of *in vitro* culture.

Experiment 4 was designed to test the hypothesis that vitrified-warmed porcine embryos pretreated by the optimized centrifugation protocol will have the potential to develop full term *in vivo* after cryopreservation. Embryos were delipidated on Day 4 and returned to culture. On Day 5, blastocysts were vitrified using either OPS or 0.25 mL mini-straws, warmed and cryoprotectants removed as described above. After warming, 50-55 embryos were then surgically transferred into the oviduct of recipient gilts. Recipients were monitored weekly by ultrasonography, and pregnant gilts were permitted to farrow.

2.7. Statistical analysis

Percentages of embryo cleavage, blastocyst development, and blastocyst hatching were analyzed by a log-linear approach after arcsine transformation [39], using the Statistical Analysis System (SAS Institute, Cary, NC, USA). For all analyses, $P < 0.05$ was considered significant.

3. Results

3.1. Thermodynamic properties of the vitrification solution

The thermodynamic characteristics of the vitrification solution are shown (Fig. 1). The vitrification solution transitioned to the glass state between -120 and -150 °C. The water melting factor was -4.88 °C. The vitrification temperature (T_g) was -127.79 °C. The subsequent negative peak (exothermic peak) was attributed to a devitrification event (T_d). The onset of devitrification event started at -109.09 °C. The melting peak (positive, endothermic peak) of the solution was approximately -31.22 °C.

3.2. Complete disassociation of lipid droplets from the blastomeres

The use of high centrifugal forces for an extended period of time allowed us to achieve complete disassociation of lipid droplets from the blastomeres in the majority of porcine embryos at the 4-8 cell stages (Fig. 2A). The lipid droplet disassociation rates were not different ($P > 0.05$) when embryos were centrifuged at either 13,000 or 16,000 g within an embryo stage group (Table 1). However, across embryo stage groups, a higher complete disassociation rate was achieved in 4-cell stage embryos compared to those at 6-8 cell stages at 16,000 g ($P < 0.05$). After 48 h of *in vitro* culture post-centrifugation, lipid droplets were still observed outside the blastomeres in most of the resulting blastocysts (Fig. 2B). The percentages of blastocysts with disassociated lipid droplets were higher than the complete disassociation rates immediately after centrifugation in all four groups (Table 1). There were no significant differences among the four groups regardless of the developmental stages at the time of centrifugation and the centrifugal forces.

3.3. In vitro survival of delipidated Day 4 embryos after vitrification

Blastocyst rates resulting from vitrified Day 4 embryos pre-centrifuged for either 12 or 30 min at 13,000 g, as determined in Experiment 1, were similar ($P > 0.05$) and were lower ($P < 0.05$) than the zona-free group (Fig. 3). However, blastocysts from vitrified groups were significantly lower than those in the non-vitrified control group.

3.4. In vitro survival of vitrified blastocysts delipidated on Day 4

Day-5 blastocysts resulting from embryos, delipidated on Day 4, were able to survive vitrification in closed 0.25 mL straws at a rate not significantly different from those cryopreserved by OPS vitrification at every category: full and partial recovery (Fig. 4). The total survival rate (full recovery and partial recovery) of embryos vitrified in 0.25 mL straw vitrification was also not different than those embryos vitrified in OPS vitrification. Compared to the non-vitrified control, however, survival rates of blastocysts vitrified in either method were lower ($P < 0.05$; both full recovery and total survival).

3.5. Embryo transfer

A portion of the embryos vitrified by either OPS or closed 0.25 mL straws retained their developmental potential after ET (Table 2). Three pregnancies and one litter (six piglets) resulted from four ETs of embryos cryopreserved by OPS vitrification. Transfer of embryos cryopreserved in closed 0.25 mL mini-straws to four recipients resulted in two pregnancies and two litters (with two and five piglets, respectively).

4. Discussion

Successful banking of embryos from swine models of biomedical importance and international exchange of swine genetics in the form of cryopreserved embryos requires that the embryo must have an intact zona pellucida and is stored in a closed system to eliminate the potential of pathogen transmission [4-6]. In the present study, we demonstrated that the cryopreservation medium we used for embryo cryopreservation vitrified at -127.79°C , and that IVP porcine embryos with intact zona pellucida could be vitrified using this solution in a zona-intact state in a closed system (0.25 mL mini straws) after a simplified centrifugation-based mechanical delipidation method. Using this approach, intracellular lipids in 4-8 cell porcine embryos were completely disassociated from embryonic cells by extended centrifugation at high centrifugal force. When embryos centrifuged for 30 min were vitrified immediately after centrifugation, their cryosurvival was not different ($P > 0.05$) from those centrifuged for 12 min. When the delipidated embryos were vitrified at the blastocyst stage, there was no difference between embryos vitrified using either OPS or 0.25 mL straws ($P > 0.05$). A portion of these vitrified blastocysts also developed to full term after surgical ET into synchronized recipients.

4.1. Porcine embryos require cell-type specific treatments for their cryopreservation

Due to their extreme sensitivity to sub-physiological temperatures, porcine embryos were very difficult to cryopreserve using conventional methods [8]. The primary cause is their high level of intracellular lipids [9,29]. Traditional methods, such as slow cooling or vitrification in closed 0.25 mL mini-straws, without any pre-treatment, resulted in little success in the cryopreservation of porcine embryos [8]. Recent advances in porcine embryo cryopreservation have relied on two technologies: centrifugation-micromanipulation based mechanical delipidation [10], and vitrification at ultrarapid cooling rates [14,15,26]. In the mechanical delipidation method, intracellular lipids were first polarized by centrifugation; thereafter, the polarized lipid droplets needed to be removed by micromanipulation, especially when a slow cooling protocol was used [28,29]. The zona pellucida of delipidated embryos was damaged during micromanipulation and thus the possibility of pathogen-transmission during storage in LN_2 was increased. Alternatively, by dramatically increasing the cooling rate and employing special cryogenic devices, non-pretreated and zona-intact porcine embryos at the morula and blastocyst stages were successfully vitrified [26,27,40]. However, this approach was limited to cryopreservation of porcine embryos derived *in vivo* at the morula and blastocyst stages. Earlier stage, *in vivo* derived embryos cryopreserved using this approach resulted in limited success, as assessed by *in vitro* culture [24].

Mechanical delipidation in combination with vitrification using high cooling rates provided better protection of porcine embryos against chilling injury. This approach was first achieved in porcine embryos derived *in vivo* [16,19] and later in those generated *in vitro* [11-13]. *In vitro*-derived porcine embryos were even more sensitive to chilling injury than those derived *in vivo*, due to the suboptimal *in vitro* production system; those embryos, without prior delipidation, were difficult to cryopreserve even when vitrification at high cooling rates was employed [30,41,42]. The combined use of mechanical delipidation and vitrification at high cooling rates resulted in live births from cryopreserved IVP porcine embryos by either somatic cell nuclear transfer, or IVF using various cryogenic devices [11-13].

4.2. The cell-type specific treatments increase the likelihood of pathogen transmissibility

For banking swine models of biomedical importance, or for international exchange of swine genetics in the form of cryopreserved embryos, two requirements usually have to be met: (1) embryos must have an intact zona pellucida; and (2) be cryopreserved in closed cryostorage

devices, such as 0.25 mL mini-straws. Previous approaches for cryopreservation of porcine embryos, except embryos at the peri-hatching stages, either damaged the zona pellucida or used open systems permitting direct contact of embryo-containing media to LN₂. Both approaches have the drawback of increased transmissibility of pathogens.

Non-invasive mechanical delipidation without damaging the zona pellucida was used for the delipidation of late stage embryos [16,18]. At those stages, lipid droplets still remain in close contact with embryonic cells after centrifugation. A modification to this method for complete disassociation of externalized lipid droplets used trypsin to partially digest the zona pellucida in order to enlarge the perivitelline space [30]. An improvement to this non-invasive method was the use of saline solution with hyper-osmolality to shrink embryonic cells to obtain complete separation of lipid droplets; with this method, live births were obtained [13]. However, porcine embryos were all cryopreserved using vitrification at high cooling rates [13,16,18,30].

4.3. Porcine embryos can be cryopreserved with intact zona and in a closed system after a modified cell-type specific treatment

In those experiments, we demonstrated that by simply increasing both the centrifugal force and time, we achieved complete separation of intracellular lipids from blastomeres of 4-8 cell stage porcine embryos, and that the separated lipids lost their ability to migrate back into the cytoplasm of blastomeres during subsequent culture, even in those with lipid droplets being associated with blastomeres through string-like structures in some embryos. Perhaps association areas of lipid droplets with the blastomeres were too small to allow migration of the lipid droplets back into the blastomeres. Porcine embryos delipidated by this method survived cryopreservation comparable to those centrifuged at 13,000 g for 12 min. Zona removal further improved *in vitro* survival of vitrified/warmed embryos. If delipidated embryos were allowed to develop into blastocysts, those blastocysts survived cryopreservation in a closed system at a comparable rate to those vitrified at ultrarapid cooling rates. A proportion of those blastocysts had the ability to develop to healthy piglets with normal birth weights following surgical transfer into synchronized surrogates. In comparison with ET results by other groups using non-invasive mechanical delipidation approaches, our efficiencies resulting from embryos vitrified by open (six live births from 201 embryos) and closed (seven from 212) systems seemed similar to Beebe *et al.* achieved in embryos (five from 147) derived *in vivo*, although they were slightly lower than Li *et al.* in IVP embryos (12 from 175).

5. Conclusion

In conclusion, in these experiments, complete disassociation of lipid droplets from blastomeres of cleavage stage porcine embryos was achieved using high centrifugal forces for an extended interval. The disassociated lipid droplets in the majority of centrifuged embryos lost their ability to migrate back into embryonic cells. Embryos delipidated by this method survived vitrification in conventional closed mini-straws, and some developed to term after being transferred into synchronized surrogates. These results have important implications for banking swine models and international exchange of swine genetics as cryopreserved embryos.

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Fig. 1. Thermodynamic properties of the vitrification solution used in this experiment. Tg = vitrification temperature; Td = devitrification temperature; Tm = melting temperature.

Fig. 2. Delipidation of Day 4 porcine embryos by centrifugation: complete disassociation of intracellular lipids after centrifugation (a); externalized lipid droplets outside blastomeres at 48 h after centrifugation (b); embryos immediately after warming showing externalized lipid droplets (c); and vitrified blastocysts 24 h after warming (d). The scale bars in (a) and (b) represent 20 μm , and in (c) and (d) represent 50 μm .

Fig. 3.

Cryosurvival of cryopreserved porcine Day 4 embryos using OPS vitrification after 48 h of culture as assessed by the formation of blastocysts. Control was non-centrifuged and non-vitrified embryos. A portion of warmed embryos were treated with 0.1% pronase to remove their zona pellucida immediately after warming (zona-free group). Values in parenthesis indicate number of embryos (three replications).

^{a,b}Columns without a common superscript differed ($P < 0.05$).

Fig. 4.

In vitro survival of porcine embryos vitrified at blastocyst stage after delipidation using centrifugation at 13,000 g for 30 min at 4-8 cell stages. Number of blastocysts in each treatment group is shown in parenthesis (three replications).

^{a,b}Within each category, columns without a common superscript differed ($P < 0.05$).

Table 1

Porcine embryos with disassociated lipid droplets from embryonic cells after centrifugation at various centrifugal forces for 30 min, and the resulting Day-6 blastocysts with lipid droplets remaining outside the embryos.

Treatment	Total embryos (replicates)	Embryos w/ disassociated lipid droplets	Embryos w/ disassociated lipid droplets (% ± SEM)	Blastocysts (% ± SEM)	Blastocysts w/ disassociated lipid droplets (% ± SEM)
4-cell, 13,000 g	38 (3)	29 (76.32 ± 2.57) ^{ab}		14 (36.84 ± 3.38)	12 (85.71 ± 7.64)
4-cell, 16,000 g	59 (3)	50 (84.75 ± 6.72) ^a		16 (27.12 ± 3.92)	15 (93.75 ± 4.17)
6-8-cell, 13,000 g	82 (3)	51 (62.20 ± 3.17) ^b		23 (28.05 ± 2.17)	21 (91.30 ± 7.43)
6-8-cell, 16,000 g	71 (3)	46 (64.79 ± 3.95) ^b		23 (32.39 ± 1.98)	22 (95.65 ± 6.67)

^{a,b} Within a column, means without a common superscript differ ($P < 0.05$).

Table 2

Results after transfer of vitrified-thawed porcine embryos.

Surrogate	Vitrification	No. embryos	Pregnancy status	Pregnancy (d)	No. piglets
1	OPS	54	-	0	0
2	OPS	52	+	40	0
3	OPS	50	+	Full term	6
4	OPS	45	+	84	0
5	0.25 mL straws	50	+	Full term	2
6	0.25 mL straws	52	+	Full term	5
7	0.25 mL straws	55	-	0	0
8	0.25 mL straws	55	-	0	0