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Birth of Piglets After Transfer of Embryos Cryopreserved by Cytoskeletal Stabilization and Vitrification

J.R. Dobrinsky,[1,2] V.G. Pursel,[3] C.R. Long,[2] and L.A. Johnson[2]

Germplasm & Gamete Physiology Laboratory[2] and Gene Evaluation & Mapping Laboratory,[3] Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

ABSTRACT

Pig embryos suffer severe sensitivity to hypothermic conditions, which limits their ability to withstand conventional cryopreservation. Research has focused on high lipid content of pig embryos and its role in hypothermic sensitivity, while little research has been conducted on structural damage. Documenting cytoskeletal disruption provides information on embryonic sensitivity and cellular response to cryopreservation. The objectives of this study were to document microfilament (MF) alterations during swine embryo vitrification, to utilize an MF inhibitor during cryopreservation to stabilize MF, and to determine the developmental competence of cytoskeletal-stabilized and vitrified pig embryos. Vitrified morulae/early blastocysts displayed MF disruptions and lacked developmental competence after cryopreservation; hatched blastocysts displayed variable MF disruption and developmental competence. Cytochalasin-b did not improve morula/early blastocyst viability after vitrification; however, it significantly ($P < 0.05$) improved survival and development of expanded and hatched blastocysts. After embryo transfer, we achieved pregnancy rates of almost 60%, and litter sizes improved from 5 to 7.25 piglets per litter. This study shows that the pig embryo cytoskeleton can be affected by vitrification and that MF depolymerization prior to vitrification improves blastocyst developmental competence after cryopreservation. After transfer, vitrified embryos can produce live, healthy piglets that grow normally and when mature are of excellent fecundity.

INTRODUCTION

Methods exist to adequately preserve germplasm and embryos from genetically superior animals of most of our livestock species except the pig. The use of embryos in addition to sperm represents a potential increase in the efficiency of transmitting improved genetic potential. While cryopreservation of boar sperm has been commercially applied since 1975, little success has been realized in preserving pig oocytes and embryos. Pig embryos suffer from severe sensitivity to hypothermia [1–4], which limits their ability to withstand conventional preservation. Research has focused on the high lipid content of pig embryos and its role in hypothermic sensitivity and cryosurvival. Innumerable studies have been reported on the conventional freezing of pig embryos, while vitrification shows promise of eluding the dangers associated with cooling sensitivity and ice crystallization [5].

Vitrification is the rapid cooling of a viscous, solute-concentrated liquid medium in the absence of ice crystal formation. An amorphous glass forms during rapid cooling, usually by direct submersion into liquid nitrogen of a plastic straw containing the embryo suspension. The glass retains the normal molecular/ionic distributions of a liquid but remains in an extremely viscous, supercooled form [6]. The glass is devoid of all ice crystals, and embryos are not subjected to cellular damage that can be associated with ice crystal formation [7]. Cattle embryos have been successfully cryopreserved with vitrification [8, 9]. More recently, van Wagtendonk-de Leeuw et al. [10] showed that bovine embryo vitrification can be successfully applied under field conditions without a reduction in pregnancy rate. Dobrinsky and Johnson [11] first demonstrated the efficacy of vitrification of swine embryos where survival and subsequent development in vitro could be established (~40%); however, survival was limited to expanded and early hatched blastocyst-stage embryos. Vitrification has been shown to be able to support development of cryopreserved embryos to term [12, 13]; however, these studies involved low numbers of recipients, and the work has not been repeated.

Cryopreservation can be extremely disruptive to the cellular organization of embryos (reviewed in [14]). Ice crystal formation can lyse plasma membranes; storage in liquid nitrogen can disrupt critical intracellular functions and organelles; and the central cytoarchitecture of a cell can be altered or destroyed. The objectives of this study were to document the disruption of the embryonic cytoskeleton during vitrification of pig embryos and to determine the ability of the microfilament (MF) inhibitor cytochalasin-b to prevent irreversible disruption to MF and prevent plasma membrane disruption, while testing the developmental competence of cytoskeletal-stabilized and vitrified pig embryos in vitro and in vivo with the repeatable production of live offspring after cryopreservation and embryo transfer.

MATERIALS AND METHODS

Experimental Design

Pig morulae/early blastocysts (MB), expanded blastocysts (XB), or hatched blastocysts (HB1 = < 400-μm diameter; HB2 = > 400-μm diameter) were cryopreserved by vitrification in the presence or absence of the known MF inhibitor, cytochalasin-b (cyto-b) (Fig. 1). After vitrification, treated and control embryos were diluted/rehydrated in the absence of cyto-b. Embryos were placed into standard culture medium (BECM-3 [15]) to monitor postthaw developmental competence in vitro or were surgically transferred via transoviductal uterine catheterization into asynchronous (−24 h) surrogate females. Some embryos were processed for immunocytochemical staining of MF, microtubules (MT), and nuclear chromatin before, during, and after cryopreservation to monitor subcellular changes during cryopreservation.

[1]Correspondence: J.R. Dobrinsky, USDA-ARS, Germplasm & Gamete Physiology Laboratory, BARC-East, Bldg. 200, Rm. 22, Beltsville, MD 20705-2350. FAX: 301 504 5123; e-mail: bigjohn@lpsi.barc.usda.gov

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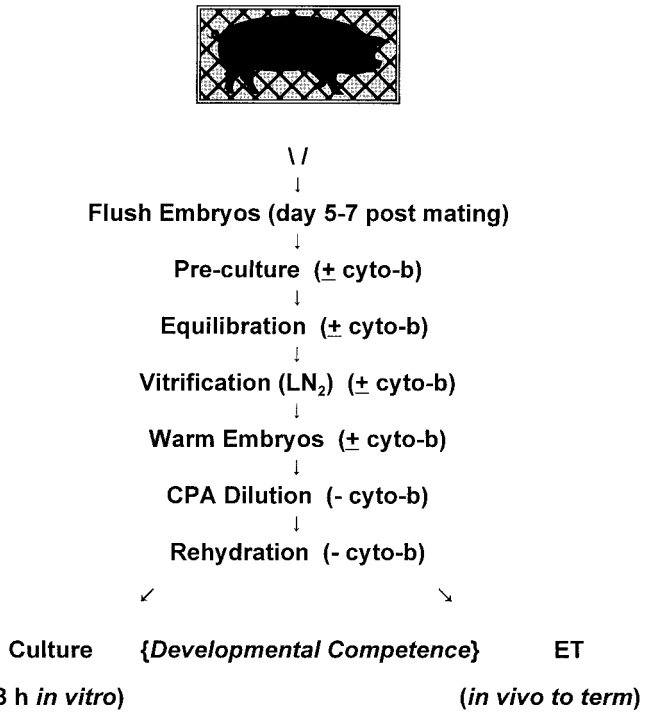

FIG. 1. Flow diagram of the vitrification protocol marking the presence or absence of cyto-b during or after cryopreservation.

Embryo Recovery and Culture

Embryos for this study were produced from natural, normal estrous cycle crossbred gilts that were 6 mo of age or older and that weighed at least 100 kg at the time of use. All gilts were artificially inseminated twice during the period of standing estrus (~24 h) with freshly collected and pooled semen that was diluted in Beltsville-TS [16] to a concentration of 5×10^9 spermatozoa for each 80-ml dose. Gilts were slaughtered 5–7 days after onset of estrus (onset = Day 0). Reproductive tracts were excised from the gilts within 3–5 min of slaughter, maintained at 38°C, and transported 200 yards to the laboratory. The tracts were prepared for flushing by trimming the oviducts and uteri away from the connective tissue while maintaining tissue temperature at 38°C. Embryos were recovered by flushing the excised oviducts and uterine horns with warm (38°C), sterile, pH-balanced BECM-3 [15] supplemented with 10% fetal bovine serum (FBS). The only embryos used were those embryos recovered from gilts slaughtered on Days 5, 6, and 7 that were MB, XB, or HB, respectively, and were graded as excellent or good for developmental stage and morphological appearance. After recovery, all embryos were randomly distributed across all treatments within a given experimental design. Embryos were cultured in Nunc (#176740; Roskilde, Denmark) 4-well tissue culture plates, 500 μl BECM-3/10% FBS culture medium per well, with the inner reservoir filled with 2 ml BECM-3/FBS. For long-term culture, embryos were washed four times in the inner reservoir in four distinct and separate locations; they were then washed once each in three of the wells and next placed into the fourth and final well to reside in culture. Embryos were cultured at 38.7°C in 5% CO_2 in humidified air throughout the study. MB were cultured for 72 h posttreatment in vitro; XB were cultured 48 h posttreatment in vitro; HB were cultured 24 h posttreatment in vitro.

A second trial was conducted to further validate the effectiveness and repeatability of producing live offspring after transfer of vitrified/warmed HB (see Table 4). Nonsuperovulated, naturally cycling hybrid gilts were artificially inseminated and slaughtered as described above. Embryos were recovered as described above on late Day 6 (estrus onset = Day 0) as XB or early HB and were cultured for 1–8 h until embryos reached 325- to 375-μm (diameter) hatched blastocysts (HB1). All HB1 embryos graded as excellent or good for developmental stage and morphological appearance were vitrified.

TABLE 1. Progressive development in vitro of morphologically different stages of swine embryos after cryopreservation by vitrification under the influence of microfilament stabilizer cyto-b.

Stage of development*	mVS3a Control			mVS3a + Cyto-b		
	n	Dev	% Dev	n	Dev	% Dev
MB	17	0	0[a]	17	1	6[a]
XB	27	6	22[ab]	25	15	60[bZ]
HB1 (<400 μm)	36	10	28[b]	48	43	90[cZ]
HB2 (>400 μm)	14	4	29[ab]	22	9	41[b]

[a–c] Values with different superscripts within individual columns are significantly different ($P < 0.05$); ANOVA for GLM, utilizing Waller-Duncan K-ratio *T*-Test, Duncan's Multiple Range Test, and Tukey's Tests for embryonic development.
[Z] Value with a superscript within individual row is significantly different ($P < 0.01$); Chi-Square analysis.

MF Stabilization

After recovery from females and preliminary culture, treated embryos were incubated at 38.7°C in 5% CO_2 and air, 95% humidity, for 45 min in BECM-3 containing 7.5 μg/ml cyto-b (Sigma Chemical Co., St. Louis, MO; C-6762; stock: 5 mg/ml 95% food grade EtOH). Embryos were vitrified in the absence (vitrification control) or presence of cyto-b, warmed, and then processed through dilution and rehydration in pH-balanced BECM-3. Cyto-b-treated embryos were diluted and rehydrated without cyto-b to wash/remove the MF inhibitor from the embryos during osmotic rehydration. Following cryopreservation, embryos were placed into culture or transferred to surrogate females.

Embryo Cryopreservation

Embryos were vitrified as described by Dobrinsky and Johnson [11] in 6.5 M glycerol, 6% BSA-V solution. Embryos were fixed for nuclear and cytoskeletal analysis during various stages before, during, and after cryopreservation: specifically, after embryo recovery and preculture (control); after cryoprotectant equilibration; immediately after thawing; after dilution, rehydration, and 2-h culture (RM-2H); and after 24 h culture. Embryos were fixed for nuclear and cytoskeletal analysis as described below.

Developmental Appraisal In Vitro

MB were cultured for 72 h posttreatment in vitro, XB for 48 h posttreatment in vitro, and HB for 24 h posttreatment in vitro. After culture, embryos were morphologically evaluated for their developmental progression (Table 1). Embryos that had cavitated with distinct differentiation of cells into trophectoderm and inner cell mass and that possessed a normal or thinning zona pellucida were considered to be developed blastocysts or expanded blastocysts, respectively. Those blastocysts that possessed cells extruding through a ruptured zona pellucida or without a zona pel-

TABLE 2. Development of fresh, recovered, hatched blastocyst swine embryos after synchronous embryo transfer.

Recipient gilt #	Days	Embryo development			
		ET*	Fetuses	Normal	% Normal
A	≤30	16	10	8	50
B	≤30	13	12	12	92
C	≤30	13	0	—	0
D	≤30	17	10	9	53
E	≤30	14	7	7	50
5 Total		73	39	36	49
4/5 (80%) preg.		60	39	36	60

* ET, Number of embryos transferred.

lucida at all and exhibited a normal or expanding blastocoelic cavity were considered HB. Since all morulae would have to develop into blastocysts within 24 h after vitrification, those that did were considered as survived and developing in vitro. Morulae that were lysed (verified by propidium iodide staining; PI, 4 μg/ml) or did not develop into blastocysts were considered as not having survived treatment and did not develop in vitro.

During vitrification, all blastocyst-stage embryos (expanded or hatched) lose morphological representation of a blastocoele during cryoprotectant equilibration and dehydration. All lysed embryos (verified by PI staining), as well as those that did not reform blastocoelic cavities after cryopreservation/rehydration and subsequent culture, were considered as not having survived treatment and did not develop in vitro.

Developmental Appraisal In Vivo

A preliminary study of in vivo development was performed to examine nontreated, noncryopreserved, freshly recovered hatched blastocyst development after synchronous embryo transfer (Table 2). Five pairs of females in estrus on the same day were selected. One female from each pair was mated and the other left unmated. On Day 7 postestrus, when hatched blastocyst-stage embryos were developed, embryos were recovered from the mated females and transoviductally transferred within 30 min into a single uterine horn of the unmated surrogate female. On Days 28–30 of presumptive gestation, surgery was performed, pregnancy was diagnosed, and fetal development was observed.

In our initial embryo cryopreservation and transfer trial, cytoskeletal-stabilized and vitrified HB1 embryos to be transferred to surrogate females were warmed, and cryo-

TABLE 3. Development of cytoskeletal stabilized and vitrified hatched blastocyst swine embryos after embryo transfer (initial embryo transfer trial).

Recipient	Embryos		Embryo development
	# ET	# Quality*	
F	29	—	4 Fetuses Day 25
G	33	—	0 Fetuses Day 25
H	33	16	5 offspring
I	33	17	open
J	32	19	5 offspring
K	29	17	open
L	30	12	open
5 Total	157	81 (52%)	10 offspring (5.0 Litter average)

* Number of excellent–good embryos at the time of transfer, 3–5 h post-rehydration.

TABLE 4. Development of cytoskeletal stabilized and vitrified hatched blastocyst swine embryos after embryo transfer (validation embryo transfers trial).

Recipient	Embryos		Embryo development
	# ET	# High quality*	
M	32	26	10 offspring
N	33	27	open
O	31	20	open
P	31	19	10 offspring
Q	32	20	6 offspring
R	33	16	open
S	32	15	3 offspring
7 Total	224	143 (64%)	29 offspring (7.25/litter)

* Number of excellent–good embryos at the time of transfer, 3–5 h post-rehydration; remainder of embryos transferred were marginally developing, exhibiting some cellular disruption but maintaining recavitation.

protectant dilution and cellular rehydration were performed in the absence of cyto-b. Embryos were cultured in BECM-3/FBS for 3–5 h, after which recavitating embryos with advanced development were considered viable. All embryos recovered after cryopreservation were morphologically evaluated and transferred by transoviductal uterine catheterization to asynchronous (−24 h) surrogate gilts (Table 3). Recipients were checked by boar daily for manifestation of estrus. Recipients that did not return to estrus were allowed to carry the pregnancy to term, while recipients that returned to estrus for two consecutive estrous cycles of normal length were considered open or not pregnant.

The aim in our second in vivo development trial was to validate effectiveness and repeatability of producing live offspring after transfer of vitrified/warmed embryos (Table 4). Nonsuperovulated, natural cycling hybrid gilts were artificially inseminated and slaughtered as described above. Embryos were recovered as described above on late Day 6 (estrus onset = Day 0) as expanded blastocysts or early hatched blastocysts and cultured for 1–8 h until embryos reached 325- to 375-μm (diameter) hatched blastocysts (HB1). All HB1 embryos graded excellent or good for developmental stage and morphological appearance were vitrified. After warming and recovery, all embryos were cultured for 3–5 h. Only morphologically excellent to good recavitating embryos with advanced development were transferred by transoviductal uterine catheterization into asynchronous (−24 h) recipient gilts. Recipients were checked by boar daily for manifestation of estrus. Recipients that did not return to estrus were allowed to carry the pregnancy to term, while recipients that returned to estrus two consecutive cycles of normal length were considered open.

Embryo Fixation

Embryos were permeabilized, fixed, and extracted for 30 min, at 37°C, in a cytoskeletal-stabilizing buffer-extraction fixative (MTSB-EF: 0.1 M PIPES, 5 mM $MgCl_2$, 2.5 mM EGTA, 0.01% aprotinin, 1 mM dithiothreitol, 50% deuterium oxide, 1 μM Taxol, 0.1% Triton X-100, and 3.7% formalin) before, during, or after treatment. Fixed embryos (≥ 20 per treatment group) were briefly rinsed in PBS with 1% sodium azide (PBS-azide) and then stored at 4°C in PBS-azide for up to 4 wk until immunocytochemical staining. After embryos were simultaneously fixed and permeabilized in MTSB-EF, they were washed three times, 10 min each wash, at 25°C in MTSB without formalin. After wash-

ing three times in PBS supplemented with 0.4% polyvinylpyrrolidone (PVP), embryos were stored by treatment group at 4°C in PBS/PVP containing 0.02% sodium azide until processed for immunolabeling.

Fluorescence Immunocytochemical Staining of MF, MT, and Chromatin

After fixation, embryos were processed for triple-fluorescence staining for MF, MT, and chromatin. Embryos were briefly washed in PBS with 0.1% Tween 20 (PBS-Tw) and then moved to blocking solution (PBS with 2.0% BSA-V, 2.0% normal goat serum, 2.0% nonfat dry milk, and 0.15 M glycine) for 30 min; this was followed by additional PBS-Tw washes. The embryos were sequentially labeled with affinity-purified mouse anti-α/β tubulin (Sigma T-5168, T-5293; 1:100 in PBS-Tw) by incubating at 37°C for 2 h or overnight at 4°C in a 1:1 ratio of α- and β-tubulin monoclonal antibody. Embryos were washed for a minimum of 1.5 h in PBS-Tw and incubated with secondary antibody label with affinity-purified goat anti-mouse IgG-fluorescein isothiocyanate (Sigma; 1:200 in PBS-Tw) at 37°C for 2 h to stain α/β tubulin (MT). Embryos were then washed a minimum of 2 h in PBS-Tw. MF were stained by incubating the embryos in PBS-Tw with phalloidin-Texas Red X (10 μ/ml; Molecular Probes, Eugene, OR) at 37°C for 45 min. All embryos were mounted on glass microscope slides in PBS with 20% glycerol, 100 mg/ml Dabco (Sigma), and 5.0 μg/ml Hoechst 33342 (Sigma). Samples were gently compressed with a coverslip and sealed with clear nail polish. Slides were stored in the dark at 4°C until confocal microscopical analysis.

Microscopy and Image Processing

Immunolabeled embryos were observed using a laser scanning confocal microscope (LSM 410; Carl Zeiss, Oberkochen, Germany). Fluorochromes were excited using appropriate combinations of excitation and barrier filters and an argon/krypton laser for Texas Red X-phalloidin conjugates. For consistency, valid relative comparisons, and repeatability for image analysis, all images in this study were obtained using a Zeiss confocal 40×—1.3 n.a. water objective with a 2× zoom software setting. The fluorochrome was scanned individually and was digitally recombined into color images using LSM software (Carl Zeiss). Digitized images were recorded on optical disks and printed using a color dye-sublimation printer (NP-1600 Photographic Network Printer; Codonics, Middleburg Heights, OH).

Statistical Analysis

All statistics were determined by ANOVA for General Linearized Models (Duncan/Waller test, further verified with Tukey's test) with the aid of a Statistical Analysis Systems statistical software package [17].

RESULTS

Embryo Development In Vitro

Only one MB survived cryopreservation treatment, with or without cyto-b (Table 1). This embryo was of poor morphology and doubtful for in vivo developmental competence. Expanded (XB) and hatched blastocysts (HB1, HB2) exhibited low rates of survival after vitrification (22–29%; $P < 0.05$). However, vitrification under the influence of cyto-b significantly improved survival of XB and HB (< 400 μm, HB1; $P < 0.05$). Although cyto-b-treated XB had improved viability after vitrification, XB development was still significantly ($P < 0.05$) lower than for HB1 embryos. Also, cyto-b did not significantly ($P > 0.05$) improve HB2 development after vitrification.

Embryo Development In Vivo

In the preliminary in vivo trial analyzing embryo development after transoviductal uterine transfer of fresh recovered embryos, four of five surrogate females maintained pregnancy and exhibited normal fetal development (Table 2). Of 60 embryos transferred to the pregnant surrogate females, 36 (60%) normal fetuses were recovered, showing a decline in noncryopreserved hatched blastocyst development after transfer.

In the initial embryo cryopreservation trial, cytoskeletal-stabilized and vitrified embryos were transferred (29–33 per female) to 7 surrogate females (Table 3). Two surrogate females were examined on Day 25 of presumptive gestation; one recipient had 4 normal fetuses similar in morphology to fetuses from the control transfers described above. Of the remaining 5 surrogate females, 2 maintained pregnancy after embryo transfer, and each delivered 5 live and normal offspring. Piglets born after transfer of the cytoskeletal-stabilized and vitrified embryos were normal in appearance and had birth weights within the normal range for these lines of pigs. The surrogate mothers weaned 8 of the 10 offspring, which grew normally as assessed by growth rates and phenotypes; and no anatomical abnormalities were observed by macroscopic examination after weaning at 28 days of age. In these first trials, 2 of 5 recipients of stabilized/vitrified embryos farrowed the first reported live offspring produced after transfer of pig embryos cryopreserved by vitrification [12]. Of these mature offspring, 2 boars and 2 gilts have successfully proven their fecundity: the boars each inseminated 3 gilts, and the 2 gilt offspring were mated. All females farrowed normal litters.

In the validation embryo cryopreservation trial (Table 4), all embryos, after warming and recovery, were cultured for 3–5 h. Only morphologically excellent to good recavitating embryos with advanced development were transferred by means of transoviductal uterine catheterization with surgical embryo transfer. Of 7 recipient surrogate females, 4 farrowed 29 live offspring, with litter sizes of 10, 10, 6, and 3. Select offspring from these litters have been raised to maturity for study of their fecundity. Stabilized vitrification is a viable method for the long-term preservation of pig embryos—a first for maternal genetics in swine.

Immunolabeling of Embryos

Morula/blastocyst control embryos. Heterogeneous staining of MF and MT was observed between embryos and individual blastomeres within embryos, while inner cell mass and trophectodermal cells were distinguishable. Phalloidin staining of MF was localized at adjacent cell borders, and MT were organized in a perinuclear/periinclusion (lipid) pattern.

Morula/blastocyst cyto-b-treated embryos (noncryopreserved controls). No actin labeling was observed, indicating that MF were depolymerized, intermittent, and localized in multicell borders. No difference was seen in MT pattern as compared to that of controls, indicating that cyto-b had no effect on MT polymerization.

Morula/blastocyst vitrified embryos. During subsequent rehydration and 2-h culture, all embryos had some lysed

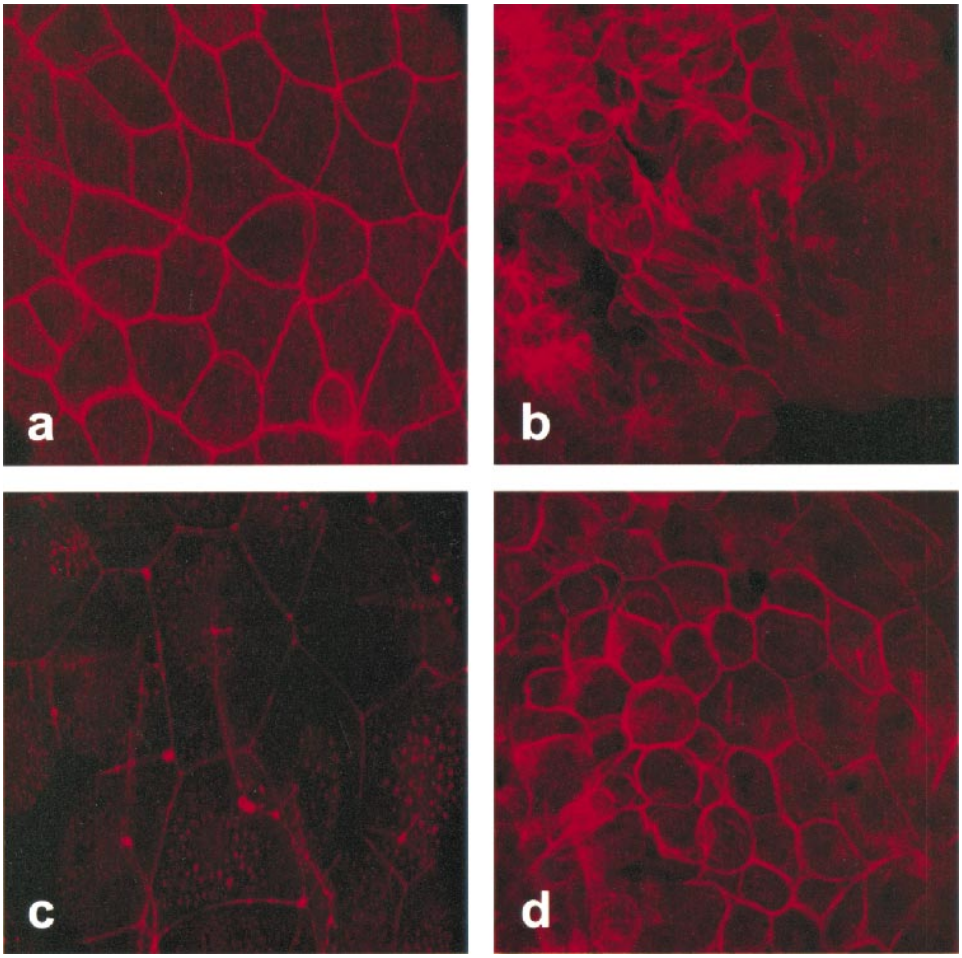

FIG. 2. Laser scanning confocal micrograph of fresh or cryopreserved pig hatched blastocysts. **a**) Control embryo after recovery from a female and immediate placement into culture, exhibiting Texas Red X-phalloidin-stained filamentous actin that shows intense MF localization at adjacent cell borders; **b**) vitrified-rehydrated embryo, 2.5 h in culture after recovery from vitrification. Localized areas of MF failed to repolymerize in any organized pattern, reflecting loss of intact plasma membranes, disrupting embryo morphology and plasma membrane integrity; **c**) fresh embryo 45 min after incubation with cyto-b; note the lack of phalloidin staining of MF, indicating MF depolymerization; **d**) cytoskeletal-stabilized (cyto-b) vitrified-rehydrated embryo, 2.5 h in culture after recovery from vitrification. Note the appearance of MF repolymerization similar to the patterning observed from control embryos.

cells, disaggregation, and nuclear disruption. MF failed to repolymerize in any organized manner, while many nuclei remained crenated. MT fluorescence intensity was reduced in most cells.

Morula/blastocyst cyto-b-treated, vitrified embryos. Cellular disruption was similar to that seen in vitrified, non-cyto-b-treated embryos. MF and MT fluorescence intensity was extensively reduced, and the cytoskeleton had no organizational integrity.

Hatched blastocyst control embryos (Fig. 2a). Embryonic cell size was reduced and lipid was mostly depleted, as reflective of increased cell number and less cell volume in comparison to those for morulae, blastocysts, and expanded blastocysts. Contiguous cell borders stained intensely for MF, and most MT (not shown) were organized in perinuclear (interphase) pattern, except in mitotic cells.

Hatched blastocyst cyto-b treatment (Fig. 2c). Cyto-b treatment clearly produced an altered pattern of actin labeling. Although cell borders were still labeled, actin also formed large clumps in the cytoplasm suggesting that the depolymerized actin may have aggregated following depolymerization or as an artifact of fixation. Tubulin (not shown) label was similar to that for controls, exhibiting long polymerized MT in interphase cells and several cells showing mitotic structures.

Hatched blastocyst, control vitrification, RM-2H (Fig. 2b). Embryos showed severe alterations in cellular structure. Overall the embryo lacked the classic morphology and cellular integrity of a blastocyst. The blastocoele cavity was collapsed, and the trophectodermal layer showed multiple invaginations. Actin labeling coalesced at cellular junctions of 3–4 cells in large patches. MT label in all cells was similar to interphase patterning, with no mitotic structures present. Large areas of the embryo lacked significant label of actin or tubulin but nuclei were present, suggesting total cytoplasmic disruption in these areas. Also, cellular cohesion was diminished in the trophectoderm layer, as evidenced by the scalloped appearance of cells along the exterior of the embryo.

Hatched blastocyst, cyto-b vitrification, RM-2H (Fig. 2d). Cyto-b treatment before and during the vitrification process prevented some of the alterations observed in non-cyto-b-treated, vitrified embryos. Labeling of MF was more uniform at the cell borders with evidence of less aggregation at junctional areas. MT label was similar to that of controls, and no mitotic structures were observed. However,

all cells were labeled, indicating less disruption of blastomeres and progressive recovery from preservation.

DISCUSSION

Documenting cellular damage during or after cryopreservation provides useful information for understanding cellular sensitivity to cryopreservation. Such information may lead to better protocols for embryo cryopreservation, improving the ability to produce live offspring after embryo transfer while providing a better understanding of domestic animal embryology. In this study, our objective was to document cellular disruption, specifically to the embryonic cytoskeleton, during and after cryopreservation in order to develop methodologies to circumvent such disruptions that would lead to the production of live offspring after cryopreservation and subsequent embryo transfer. We have documented MF damage during swine embryo vitrification and use of cytochalasins prior to and during cryopreservation to help deter damage and stabilize the plasma membrane. This has enabled the repeatable, unprecedented production of live offspring after transfer of cytoskeletal-stabilized and vitrified/warmed embryos into surrogate females.

Some vitrification solutions have been shown to be extremely toxic to pig embryos [11, 18, 19], and structural components of the cytoskeleton react quite differently to cryoprotectant exposure [5, 20]. Under the influence of high molar concentrations of glycerol needed for vitrification, MT in morula- to hatched blastocyst-stage pig embryos retain polymerization throughout the vitrification protocol. MF are variable in their response to cryoprotectants, and the variability is directly related to embryonic stage of development. Prior to cryopreservation of morulae and early blastocyst-stage embryos, MF were disrupted by interaction with the cryoprotectants. After thawing and subsequent rehydration, cell lysis, membrane disintegration, and nuclear damage were observed. MF of control morulae and early blastocysts equilibrated in vitrification solution repolymerize normally following rehydration, while MT retain normal staining intensity and nuclei regain normal fluorescent, noncrenated morphology. Therefore, irreversible damage of morula/early blastocyst-stage pig embryos is associated with cooling to −196°C but not with the exposure to the cryoprotectants used for vitrification. In contrast, some vitrified pig hatched blastocysts retained polymerized MF and MT but exhibited regions of intact cortical MF and heterogeneous, cytoplasmic MT staining after rehydration. Other vitrified embryos revealed extensive cell lysis and perturbations of normal MF and MT localization after rehydration. Noncryopreserved hatched blastocysts equilibrated in vitrification solution exhibited normal MF repolymerization while MTs retained normal perinuclear staining patterns.

Our observations that the survival of pig embryos after standard vitrification depends upon developmental stage of the embryo confirms and expands previous reports. Simply, untreated morula/early blastocyst pig embryos do not survive cryopreservation. Treatment with cyto-b did not improve their viability after vitrification. However, cyto-b treatment provided a 3-fold improvement in survival of expanded (60%) and hatched blastocysts under 400 μm in diameter (90%). Although cyto-b-treated expanded blastocysts had improved viability after vitrification, their development in terms of blastocoele reformation and proliferation in culture after vitrification was still lower than that of hatched blastocysts. These data are the highest rates of in vitro development after cryopreservation ever attained in our laboratory, and results are consistent across embryo donors.

We utilized laser scanning confocal microscopy to illustrate cause and effect of cryopreservation under the influence of or without MF depolymerization by cyto-b, not for a quantitative analysis of how well cyto-b performs as a depolymerization agent, as much literature is already available describing the efficacy of cyto-b as such an agent. Therefore, quantitative fluorescence information was not collected or considered crucial to the production of live offspring but could be the subject of future research. Cellular analysis with laser scanning confocal microscopy revealed reduced fluorescence intensity of MF in cyto-b-treated embryos (Fig. 2c). Non-cytochalasin-treated vitrified embryos (Fig. 2b) exhibited partial or major cytoskeletal disruptions within 2 h after rehydration, whereas cytochalasin-treated vitrified embryos (Fig. 2d) mostly exhibited normal repolymerization of MF and other cytoskeletal components after vitrification. These experiments show that the cytoskeleton is affected during vitrification, and that MF depolymerization prior to cryopreservation can protect MF structures during cryopreservation while this stabilization significantly improves expanded and hatched blastocyst (HB1) development after cryopreservation. After transfer, vitrified/warmed embryos have the capability to develop to term as normal, healthy live offspring.

The cytoskeleton is a complex network of protein constituents, actin (MF) and tubulin (MT), distributed throughout the cytoplasm of a cell (reviewed in [14, 21]). These constituents impart three-dimensionality and mechanical strength to the surface of a cell and provide a system of fibers that regulate cell polarity, cell shape, cell movement, and the plane of cell division. Actin and tubulin filaments bind a variety of accessory proteins that enable them to participate in distinct functions in different regions of a cell, including the plasma membrane. Thus, this highly organized network of filaments forms an internal framework for the large volume of cytoplasm within the cell.

An intact cytoskeleton is essential for cytokinesis and karyokinesis, and if it is irreversibly disrupted, the mitotic cell cycle will cease, junctional complexes may be compromised, and solute transport systems would be affected. Maintaining the integrity of the cytoarchitecture within an embryo during cryopreservation is of the utmost importance, especially with fragile pig embryos. Because of the cooling sensitivity and fragile plasma membranes, finding ways to overcome or prevent cytoskeletal disruption would improve the survival of pig embryos during and after cryopreservation.

While the cytoskeleton is important in maintaining structural integrity within a cell, cryoprotectants and cryopreservation can damage the cytoskeleton. Permeating cryoprotectants, such as glycerol and propylene glycol, are organic solutes; and among their properties is that of acting to depolymerize MF and MT. Depolymerization may be beneficial toward protecting these cytoskeletal components during osmotic stresses induced by exposure to or removal of cryoprotectants. When coupled with vitrification, however, disruption to the MF and/or MT can be irreversible and lethal to embryos. Total disruption of the plasma membranes of individual blastomeres, as well as lack of blastocoele cavity reformation, indicates that many pig embryos cannot tolerate cryopreservation or cryoprotectant treatments and that membrane integrity and metabolic activity are jeopardized.

Cytoskeletal stabilizers such as the cytochalasins have

been used extensively in reproductive biotechnology for studies dealing with micromanipulation of embryonic development [22, 23]. It is generally accepted that cytochalasins are an MF inhibitor disrupting actin polymerization (Fig. 2) by blocking monomer addition at the fast-growing end of the polymers [24], thus preventing cytokinesis without affecting karyokinesis [25, 26]. Treatment of cells with cytochalasins makes the plasma membrane less rigid and more elastic so that MF are not disrupted during micromanipulation [22]. Cumulatively, the prevention of permanent cytoskeletal disruption and the ability to then vitrify pig embryos have tremendously improved the in vivo survival of cryopreserved pig embryos as well. Our initial in vivo development trial produced 3 pregnancies from 7 embryo transfers. Of the 5 surrogate females allowed to go to term of pregnancy, 2 farrowed 5 offspring each. For the validation trial, we changed our approach for embryo preservation and transfer with the hope of improving our ability to increase pregnancy rates and litter sizes. First, instead of recovering hatched blastocysts from Day 7 donor females and hoping to obtain HB1 embryos, we recovered XB or early HB from late Day 6 females, and then cultured the embryos 1–8 h to the desired HB1 stage (325–400 μm HB). This enabled greater use of almost all embryos recovered from donor females to be vitrified and then later recovered for embryo transfer, tremendously increasing our number of morphologically correct embryos available for cryopreservation per donor female. Preliminary results (data not shown) showed no effect of embryo culture to the HB1 stage on embryo survival and development in vitro; therefore, we employed this method of producing larger numbers of HB1 embryos for our second in vivo development validation trial. Also, to improve presumptive pregnancy rate and litter sizes, after warming, recovery, and 3- to 5-h culture prior to embryo transfer, we transferred only recavitating embryos with excellent to good morphology. From this, our pregnancy rates improved to near 60%, while litter sizes improved from 5 to 7.25 piglets per litter.

What is evident from these data, but not shown in our results, is the variability that recipient surrogate females can have in relation to establishment and maintenance of pregnancy. For example, from the validation trial, recipients M and N received embryos by surgical transfer on the same day. The embryos these females received were pooled from the same vitrified/warmed embryos recovered that day for transfer. The developing embryos were evenly segregated across the two recipients and surgically transferred the same day, less than 30 min apart from each other. Recipient M, which received 32 morphologically equivalent and developing embryos (as compared to recipient N), went on to farrow 10 live offspring. Recipient N, which received 33 morphologically equivalent and developing embryos (as compared to recipient M), returned to estrus on Day 24 of prospective gestation and was diagnosed open or not pregnant. This variability in recipient surrogate females is left unexplained and could be the subject of future research on pig embryo transfer recipient selection and management.

Stabilized vitrification is a viable method for the long-term preservation of pig embryos. As a new production tool for the swine industry, pig embryo cryopreservation will be instrumental in continuous production of animals of high genetic merit, capable of having a significant impact on the improvement of the world swine population and medicine.

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