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Effect of Dehydration Prior to Cryopreservation of Large Equine Embryos

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Abstract

Cryopreservation of equine embryos > 300 μ m in diameter results in low survival rates using protocols that work well for smaller equine embryos. These experiments tested the potential benefit of incorporating a dehydration step prior to standard cryopreservation procedures. Forty-six, d 7–8, grade 1, equine embryos $\geq$ 400 μ m in diameter were subjected to one of the following treatments: (A) 2-min in 0.6 M galactose, 10 min in 1.5 M glycerol, slow freeze (n=21); (B) 10 min in 1.5 M glycerol, slow freeze (n=15); (C) 2 min in 0.6 M galactose, 10 min in 1.5 M glycerol, followed by exposure to thaw solutions, then culture medium (n=5); (D) transferred directly to culture medium (n=5). Frozen embryos were thawed and subjected to a 3-step cryoprotectant removal. Five embryos from each treatment were evaluated morphologically after 24 and 48 h culture (1=excellent, 5=degenerate/dead). All treatments had at least 4/5 embryos with a quality score $\geq$ 3 at these time points except treatment B (2/5 at 24 h, 1/5 at 48 h). Subsequent embryos from treatment A (n=16) or B (n=10) were matched in sets of two for size and treatment, thawed, and immediately transferred in pairs to 13 recipients. Only two recipient mares were pregnant; one received two 400 μ m embryos from treatment A, and the other one 400 μ m and one 415 μ m embryo from treatment B. There was no advantage of incorporating a 2 min dehydration step into the cryopreservation protocol for large equine embryos.

Introduction

Cryopreservation of equine embryos has resulted in acceptable pregnancy rates when the embryos are smaller than 300 μ m in diameter (50–65% on average [20] and as high as 80% [19]); however, these protocols do not result in acceptable pregnancy rates for larger, more advanced equine embryos [12,19]. The value of cryopreserving equine embryos greater than 300 μ m in diameter is linked to embryo recovery rates during uterine flushes. Equine embryos flushed from the mare 6 d after ovulation are generally smaller than 300 μ m and have a higher survival rate after cryopreservation than more advanced embryos. However, the embryo recovery rate is higher when mares are flushed 7 to 8 d after ovulation, a stage at which the embryos are usually larger than 300 μ m [20]. An acceptable cryopreservation protocol for large embryos would prevent the loss of valuable embryos collected in more advanced stages of development by enabling their preservation until recipients are available, saving money for the commercial producer and making collection of embryos for research more efficient.

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Attempts to freeze large equine embryos usually are fraught with low sample numbers, and most have resulted in unacceptable pregnancy rates. Maclellan et al. [11] cryopreserved embryos between 300 and 600 μm by conventional freezing using glycerol as the cryoprotectant. Pregnancy rates of large embryos frozen with this protocol (2/5) were promising though still not comparable to frozen-thawed embryos $<300 \mu\text{m}$ (4/5). With this same cryopreservation protocol, similar pregnancy rates were observed when large equine embryos were exposed prior to cryopreservation to cytochalasin B, an inhibitor used to prevent disruption of microfilaments and plasma membrane during freezing (43%, 3/7) [12]. However, other studies have found that pre-treatment with cytochalasin-B and trypsin can cause irreversible changes that complicate embryo handling and that likely are detrimental to embryo survival [23].

Young et al. [25] tested three highly divergent cryopreservation procedures with equine embryos between 300 and 680 μm in diameter. The first treatment involved the one-step addition of 1 M glycerol before freezing and a four-step removal of glycerol post-thaw; all embryos in this treatment degenerated during culture. The second treatment included a two-step addition of 4 M glycerol followed by a step down to 2 M glycerol prior to freezing and a four-step removal of cryoprotectant post thaw; all embryos in this treatment survived cryopreservation and continued to grow during post-thaw culture with 4 of 6 maintaining an excellent quality score (QS=1). The third treatment was a standard vitrification protocol in which only 2 of 7 embryos maintained a quality score of 3 or better during culture after warming. Two of six embryos from treatment 2 resulted in pregnancy when transferred to recipient mares.

While glycerol is the usual cryoprotectant chosen for equine embryos, other cryoprotectants have been tested. Cryopreservation of 300 – 1000 μm equine embryos in 4.8% (v/v) methanol resulted in d 16 pregnancy rates of 23% (5/22), which was not as good as a 2-step glycerol addition protocol using the same freezing curve (38%, 8/21) [1]. Ethylene glycol has also been used to freeze early equine blastocysts flushed 6 days after ovulation, but with a similar d 15 pregnancy rate as glycerol (EG, 2/8; glycerol, 3/8)[6]. Pfaff [17] reported a high percentage of dissociated cells, a loss of cell volume during culture, many pycnotic nuclei, and low quality scores for large equine embryos that had been frozen in ethylene glycol. None of these characteristics was observed of embryos that had been frozen in glycerol.

The benefit of adding sucrose to cryopreservation media has been explored in experiments with embryos of other species. In addition to penetrating cryoprotectants such as glycerol, which diffuse across the cell membrane to exert cryoprotective properties intracellularly, non-penetrating molecules, such as sucrose, are used to draw water out of cells osmotically to decrease the intracellular water available for ice crystal formation. An experiment with murine zygotes demonstrated the tolerance of embryos to concentrations of mono- and disaccharides up to 1.5 M for 10 min causing a loss of 85% of cell water, yet 75% of these embryos still developed into hatching blastocysts after osmotic recovery [14]. Equine embryos may also benefit from the use of sugars to enhance dehydration prior to freezing or for preventing excessive swelling during thawing, both of which may be especially important for large embryos.

The reason(s) for the failure of large equine embryos to survive cryopreservation are unknown. Some question the permeability of the capsule, an acellular glycoprotein coating secreted by the embryo beginning approximately 6 d after fertilization, to cryoprotectants [5]. The lower surface-to-volume ratio of larger embryos also slows the rate that cryoprotectants reach equilibrium concentrations within the embryo. Day 7 and 8 equine blastocysts are characterized by a large fluid filled blastocoele, which will be slower to equilibrate during addition and removal of cryoprotectant than smaller blastocoeles. Researchers have reported finding

ruptured or exploded large equine embryos upon thawing [16], possibly indicating that these embryos were not properly dehydrated.

With these potential problems in mind, experiments were designed to test the potential benefit of incorporating a brief dehydration step by exposure of the embryos to a non-penetrating monosaccharide (galactose) prior to initiating a standard cryopreservation protocol. The hypothesis was that reducing the volume of the blastocoele would allow more rapid equilibration with cryoprotectant and increase the rate of survival post-thaw.

Materials and methods

Preliminary experiments with bovine embryos

In vitro produced, hatched bovine embryos were used for preliminary experiments because of the limited availability of equine embryos. Cumulus-oocyte complexes were aspirated from 3- to 8-mm follicles of slaughter house-derived ovaries within 4 h of slaughter. Oocytes with compact cumulus cell layers were selected under a stereomicroscope, and matured for 23 h in vitro using standard procedures [2]. Oocytes were fertilized in vitro using frozen-thawed semen centrifuged through a percoll gradient to isolate live spermatozoa. Spermatozoa were co-incubated with mature oocytes for 18 h in fertilization CDM. Fertilized oocytes were cultured to the 8-cell stage in CDM-1 and cultured in CDM-2 until hatching occurred. These procedures have been described in detail [2]. Hatched embryos were incubated in 0.3 or 0.6 M galactose in Syngro® for 10 min, and diameters measured every min using an eyepiece micrometer. This information was used to determine the galactose concentration and duration of the predehydration step to be used for equine embryos.

Embryo collection

Twenty-eight mares of light horse breeds were used as embryo donors. Mares were given eFSH (12.5 mg, i.m., Bioniche Animal Health, Bogart, GA, USA) or reFSH with the α and β chains linked covalently (0.35, 0.5, or 0.65 mg, i.m., Aspen Bio, Castle Rock, CO, USA) twice daily once follicles 22–25 mm in diameter were detected. The following day mares were administered cloprostenol sodium (250 μ g, i.m.), and FSH treatment continued until more than 50% of developing follicles reached 35 mm in diameter. Mares were allowed to ‘coast’ for 36 h prior to receiving hCG (2500 IU, i.v., Intervet), inseminated 12 h later with 500×10^6 progressively motile spermatozoa, and rebred with cooled semen the following day. Ovulation was confirmed by daily transrectal ultrasonography, and uterine flushes were performed 7 or 8 d post-ovulation as previously described [20].

Forty-six, grade 1 or 2 equine embryos were recovered. Embryos were suspended in warmed Syngro® holding medium (Bioniche Animal Health, Bogart, GA, USA) for transport 0.5 km in an opaque container to prevent exposure to sunlight. Upon arrival to the laboratory, embryos were rinsed through two drops (500 μ l each) of room temperature Syngro®.

Evaluation of embryos

Embryos were evaluated for size and morphology at a magnification of 15 or 25-fold using a stereo microscope with an eyepiece micrometer calibrated with a hemacytometer. Embryos were graded on a scale of 1 to 5, 1 being excellent, 2 good, 3 fair, 4, poor, and 5 degenerate or dead [13]. Only embryos of grades 1 or 2 were used in the experiment, and diameters of all embryos were measured at collection. Embryos were blocked into groups according to diameter: 400–600, 601–800, and >800 μ m.

Cryopreservation of embryos

Twenty embryos were subjected to one of the following treatments followed by a 48 h culture period: (A) 2 min incubation in 0.6 M galactose, 10 min incubation in 1.5 M glycerol + 0.6 M galactose, slow freeze; (B) 10 min incubation in 1.5 M glycerol + 0.6 M galactose, slow freeze; (C) 2 min incubation in 0.6 M galactose, 10 min in 1.5 M glycerol + 0.6 M galactose, followed by exposure to thaw solutions (see below), then culture medium (50:50 DMEM-Ham's F12 medium + 10% FCS); (D) transferred directly to culture medium. Diameters of embryos were measured every minute during the incubation steps of treatments A–C. Culture medium was equilibrated in 5% CO₂ in air at 38°C. Twenty-six additional embryos were subjected to treatments A or B and reserved for transfer to recipient mares.

All cryopreservation media were at room temperature and Syngro®-based (Bioniche Animal Health, Bogart, GA, USA) with the following modifications: CaCl₂ concentration was reduced by half to 0.5 mM, glycine was increased from 0.1 mM to 5 mM, NaCl was decreased to compensate for a change of 3.4 mOsm and 1/3 of the NaCl was replaced with equimolar choline chloride. These changes were based on studies showing that increased Ca concentrations were detrimental to equine blastocyst development and morphology [3], and that substitution of choline chloride for NaCl as the major extracellular cation in cryopreservation media was beneficial for survival of murine oocytes and their subsequent development [21,22]. An inverse relationship between high sodium concentrations in culture medium and blastocyst development rates has also been reported for murine zygotes [7] and in vitro produced bovine embryos [10].

Embryos to be cryopreserved (treatments A and B) were frozen individually in 0.25 ml plastic straws in a Cryologic CL-3300 controlled-rate freezer (Bioniche Animal Health, Bogart, GA, USA). During the 10 min incubation in 1.5 M glycerol + 0.6 M galactose, embryos were loaded into straws so that after exactly 10 min, the straws were inserted into a cryochamber pre-cooled to –6°C. After 2 min the straws were seeded and held at –6 °C for 10 min, followed by a decrease in temperature of 0.5°C/min until –32 °C was reached. Embryos were held at –32 °C no longer than 3 min and then plunged into LN₂.

Embryo thawing and culture

Straws were held in air for 8 sec followed by submersion in a 35°C water bath for 30 sec. Embryos were expelled from the straw into a petri dish and immediately transferred to the first of three thaw solutions. From the water bath to the first thaw solution, the elapsed time did not exceed 2 min. The following sequence of solutions was used to remove cryoprotectant in 8 min steps each: 0.6 M glycerol + 0.6 M galactose, 0.3 M glycerol + 0.6 M galactose, 0.6 M galactose. All media were Syngro® based. Following completion of cryoprotectant removal, the embryo was transferred to a culture dish well containing 1 ml of culture medium (50:50 DMEM/Ham's F-12 + 10% FCS) which served as a rinse step. The embryo was then placed into another well containing the same culture medium and incubated at 5% CO₂ in air at 38°C for 48 h; diameters and quality grades were obtained at 24 and 48 h.

Embryo thawing and transfer

Embryos transferred to recipients were transported to the transfer facility in a small liquid nitrogen dewar and thawed on site. All thawing procedures and media were performed at room temperature (average 19°C) as described above for cultured embryos except that following completion of cryoprotectant removal, embryos were placed in 1 ml of Syngro® and promptly loaded into a 0.25 mL straw for immediate transfer. Elapsed time from the completion of cryoprotectant removal to completion of the transfer did not exceed 3 min.

Five minutes prior to transfer, mares were administered acepromazine (20 mg, i.v.), flunixin meglumine (Banamine®, 500 mg, i.v., Schering-Plough), and altrenogest (Regumate®, 0.44 mg/kg, orally, Intervet). Mares continued to receive Regumate® daily until determined to be not pregnant or until termination of pregnancy. Embryos were transferred nonsurgically to uteri using a Cassou transfer instrument protected with a sterile plastic chemise. Mares were checked for pregnancy via transrectal ultrasonography on days 11, 12, 14, and 16. If an embryonic vesicle was not detected by d 16, the mare was considered not pregnant and administered cloprostenol sodium (250 µg i.m.). All transfers were performed by a single technician.

Statistical procedures

Differences in quality scores and diameters within and between treatments were analyzed using a one-way analysis of variance followed by pairwise comparisons of means using Tukey's test.

Results

Preliminary experiments with in-vitro produced bovine embryos were done to determine the optimal duration of galactose exposure period. Observable volume changes (shrinkage due to dehydration) occurred with all embryos, but the greatest decrease in diameter in the shortest amount of time occurred with embryos in 0.6 M galactose (Fig. 1). Because the majority of volume change for embryos in 0.6 M galactose occurred within 2 min, this time point was chosen for subsequent equine experiments.

Cultured embryos

Five embryos from each treatment were cultured after completion of the treatment. During treatments A–C embryo diameters were recorded every min of incubation in the various freezing and thawing solutions. Changes in volume were calculated relative to the measured diameter of the embryo at collection. Data for treatments for A and C incubated in freezing media were combined as they were exposed to the same solutions prior to exposure to thaw solutions. There were no significant differences in the size of embryos between treatments except at time 0 ($p < 0.05$, Fig. 2). Embryos in the A,C group had significantly less volume after the first 4 min of incubation compared to initial volume (min 2 on x-axis, Fig. 2). Volumes of embryos in group B declined steadily from 0 to 4 min. Data could not be collected for the full incubation period for treatments A and B as the final minutes were spent loading embryos into straws.

During removal of cryoprotectant, embryo diameters were recorded. There were no significant differences in the relative volumes of embryos at the beginning or end of incubation in these media within a treatment (Table 1); however, treatment B embryos had significantly smaller volumes during the cryoprotectant removal steps compared to the last measured diameter prior to freezing. There was also a significant difference in relative volume of embryos in treatments A and B in the final cryoprotectant removal medium (step 3). No other significant differences were found within or between groups.

These 20 embryos were also evaluated for diameter and grade after 24 and 48 h incubation after thawing and cryoprotectant removal. The distributions of embryo size and grade before treatment (at flush) and after 24 and 48 h incubation are given in Table 2. Although some embryo quality grades decreased from 24 to 48 h, many diameters increased, indicating that the embryos were surviving and continuing to grow.

There was no significant difference in mean initial diameter or grade of embryos allocated to different treatments (Table 2). Embryos in treatments A, B, and D did not show a significant increase in diameter over time; however, treatment C embryos were larger after 48 h in culture

than at the time of collection and 24 h ($P < 0.05$). Although there was no significant increase in size, all intact embryos in treatment D were larger after 24 h of culture; however, one embryo decreased in size after 48 h culture. Quality grades remained consistent for treatments C and D with no significant change in grades over the observation period. Treatments A and B had significant declines in embryo quality after 24 h (treatment A) and 48 h (treatment B) culture. Four out of five embryos in treatment B ruptured (trophoblast and/or capsule) upon expulsion from the straw during the thaw process.

Comparisons between treatments at the same time points are also represented in Table 2. Embryos receiving treatment A were smaller after 24 h culture than embryos in treatments C and D ($P < 0.05$). Likewise, embryos in treatment C were larger than those in treatment A after 48 h incubation ($P < 0.05$). Embryos in treatment C had better quality scores than embryos in treatments A and B after 24 h culture ($P < 0.05$). After 48 h culture, treatment B embryos had the lowest quality grades, but these were not significantly different from the other treatments. Photomicrographs of embryos from each of these groups after 24 and 48 h culture are presented in figure 3.

Transferred embryos

Twenty-six frozen-thawed embryos were paired based on treatment and size and transferred to 13 mares (Table 3). Pregnancies were detected in two mares. The first mare received two grade 1 embryos of 400 μm in diameter from treatment A. One 5 mm embryonic vesicle was detected on day 12 and, a heartbeat was detected on day 25 when the pregnancy was terminated with prostaglandin $\text{F}_{2\alpha}$. The second mare received two grade 1 embryos, one 400 μm and the other 415 μm , from treatment B. One 8 mm embryonic vesicle was detected on day 14, a heartbeat was detected on day 25, and the pregnancy was terminated on day 30 by uterine lavage. No pregnancies were detected after the transfer of any of the larger embryos. Some of the embryos that were transferred were noted to be ruptured upon thaw as indicated in Table 3. Pregnancy rates for treatments A and B were 6% (1/16) and 10% (1/10), respectively. The pregnancy rate for embryos $< 500 \mu\text{m}$ in diameter was 33% (2/6). The pregnancy rate of fresh embryos transferred by the same technician during the 2007 breeding season in our laboratory was 75% (44/59).

Discussion

The addition of a 2 min pre-dehydration incubation prior to exposure to glycerol did not improve pregnancy rates of transferred d 7 and 8 equine embryos. Transferred embryos ranged from 400 – 1350 μm in diameter, yet the only pregnancies achieved were from the smallest embryos transferred (400 – 415 μm). It was hypothesized that exposure to a non-penetrating sugar before incubation in glycerol would enhance removal of intracellular and blastocoele water, thereby decreasing the volume of blastocoele fluid requiring equilibration. In the preliminary observations of equine embryos, treatment B often resulted in ruptured or exploded embryos, which is a possible indication of ice formation. Upon thawing embryos for transfer, visual observation of embryos in both treatments indicated that the majority of embryos were intact and had a healthy appearance (few extruded cells, uniform texture of the embryo), although many remained pulled away from the capsule. It is possible that some of the capsules were ruptured, but because of the shriveled nature of the capsules, tears or holes were difficult to detect. Rupture of embryos upon thawing in slow cool cryopreservation protocols is rarely recorded in the literature; however, embryo rupture has been noted anecdotally and in at least one thesis [16].

One noteworthy observation of the embryos that resulted in pregnancy was that there were visual signs of blastocoele collapse. Many of the larger embryos did not have any signs of collapse beyond a slight pulling away from the capsule. This would suggest that the dehydration

protocol tested here did not adequately remove intracellular and/or blastocoelic water from the embryo prior to freezing. Although some of the largest embryos remained intact upon thawing, it may be that intracellular ice crystals disrupted the intracellular integrity of the embryo while not disrupting the plasma membrane or capsule. A longer dehydration period might have been more beneficial for the larger equine embryos as the time point was based on bovine embryos that did not approach the sizes of many of the larger equine embryos ($> 600 \mu\text{m}$). Because the smaller surface-to-volume ratio reduces the rate of cryoprotectant passage in and out of cells and the blastocoele, the dehydration period may need to be determined by the diameter of the embryo, with longer incubation times for increasing embryo diameters.

Another potentially beneficial alteration to the cryopreservation protocol used in this study is to use a slower cooling rate. Maclellan et al. [11,12] froze large equine embryos at a rate of $0.3^\circ\text{C}/\text{min}$ between -6°C and -30°C and $0.1^\circ\text{C}/\text{min}$ between -30°C and -33°C with a pregnancy rate of approximately 40% in two studies. This change in freezing rate increases the duration of the procedure from approximately 1 to 2 h, giving the larger embryos more time to dehydrate as freezing occurs. This extended freezing period may be especially advantageous for embryos larger than $450 \mu\text{m}$, as these embryos did not result in pregnancy when they were cooled at $0.5^\circ\text{C}/\text{min}$ in this study. While the tactic of predehydration seems simple, the challenge is finding a dehydration time point that adequately dehydrates the cells and keeps them dehydrated, and results in adequate equilibration of the blastocoele with cryoprotectant, without damaging the embryo by extended exposure to concentrated solutions (solution effects).

Regardless of the potential advantage of a longer dehydration period prior to exposure to cryoprotectant and/or a slower cooling rate, the methods tested here did result in a reduction of embryo volume by 45%. Approximately 20% of volume is osmotically inactive, so this reduction is 56% of the osmotically active fraction of the embryo. It is likely that further dehydration occurred within the straw during the freezing process as the extracellular fluid became more concentrated, drawing water from the cells which, in turn, draws water from the blastocoele.

Alternative approaches to freezing large equine embryos are being researched. Recently, successful vitrification of two equine embryos $> 800 \mu\text{m}$ after micromanipulation was reported [18]. Both embryos were subjected to microinjection of a cryoprotective solution, and one additionally subjected to aspiration of blastocoelic fluid prior to microinjection. Transfer of these embryos resulted in the formation of an embryonic vesicle detected on day 15 in the mare that received the embryo that had been aspirated of blastocoelic fluid and microinjected with cryoprotectant [18]. Although the embryo was resorbed by d 28, this may be a promising technique that also supports the hypothesis that inefficient removal of the blastocoelic fluid contributes to the cryopreservation failure for the large embryos. Aspiration of blastocoelic fluid prior to cryopreservation of equine embryos has been attempted before, but without the microinjection of cryoprotectant; however, transfer of these embryos also did not result in pregnancies [17].

Efforts to improve cryopreservation of porcine embryos are also focused on a micromanipulation approach. Lin et al. [9] found that embryos punctured with a microinjection pipette to induce blastocoele collapse had higher re-expansion rates after vitrification than unmanipulated controls. Several different methods to induce blastocoele collapse of human embryos followed by vitrification have resulted in acceptable pregnancy rates including direct puncture with 29-gauge needles (pregnancy rate 48%) [24], micropipetting (50%) [4], and micro-needle or laser pulse puncture (60% and 62%, respectively) [15]. Further research is needed to determine if these techniques can be used to improve the success of freezing large equine embryos.

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Figure 1. Mean relative volume changes and SEM of hatched bovine blastocysts incubated in 0.3 M (closed circles) or 0.6 M galactose (open circles) for 10 min compared to the initial volume of the embryo; n=11 for each treatment.

Figure 2.

Volume changes of equine embryos during incubation in galactose and freezing medium. Mean relative volumes $\pm$ SEM of embryos in treatments A and C (exposure to galactose prior to glycerol) are collectively represented by solid circles. Mean relative volumes $\pm$ SEM of embryos in treatment B (no exposure to galactose prior to glycerol) are represented by open circles. Time 0 is addition of cryoprotectant and -2 to 0 min represents incubation of embryos in 0.6 M galactose. Asterisk indicates a statistically significant difference in relative volume between treatments ($p < 0.05$). Solid circles $n = 10$, except 8–10 min ($n = 5$), open circles $n = 5$.

Figure 3.

Treated embryos after 24 and 48 h culture. Two embryos are shown for each treatment. Embryo diameters are given for longest measured diameter. Where there are two diameters given, the first is the diameter of the embryonic cells and the second the capsule. Treatment A, predehydration freeze; Treatment B, control freeze; Treatment C, cryoprotectant, no freeze; Treatment D, culture control.

Table 1

Relative volume of embryos (%) prior to cryopreservation and during cryoprotectant removal. Treatment A, predehydration; Treatment B, no predehydration prior to freezing; Treatment C, exposure to cryoprotectants without freezing.

	Treatment A	Treatment B	Treatment C
Prefreeze	52.8 ± 4.6	54.0 ± 8.1 ^a	53.5 ± 7.6
Thaw step 1	38.5 ± 6.7	19.1 ± 2.6 ^b	42.7 ± 8.5
Thaw step 2	36.8 ± 5.3	19.1 ± 2.6 ^b	36.9 ± 6.1
Thaw step 3	36.8 ± 5.3 ¹	15.9 ± 1.3 ^{b,2}	35.0 ± 6.6 ^{1,2}

Values are mean ± SEM of relative volumes of embryos as measured at the end of exposure to cryopreservation medium (prefreeze) and exposure to each of the media for cryoprotectant removal (thaw steps 1–3). Significant differences within a group are indicated by letters and differences between groups with numerals (p < 0.05)

Table 2Distribution of embryo grades (1–5)/diameters (μm) for embryos that were cultured in treatments.

Treatment A, Predehydration Freeze	At flush	24 h	48h
	1/500	3/200	3/330
	1/400	3/200	3/375
	1/300	2/415	1/415
	1/625	4/375*	4/375*
	1/1100	3/750	2/1080
Mean	1.0 ^a /585	3.0 ^{b,A} /388 ^A	2.6 ^{a,b} /515 ^A
Treatment B, Control Freeze	At flush	24 h	48h
	1/600	3/750*	4.5/290*
	1/410	4/580*	4/830*
	1/700	4/X*	4/875*
	1/950	3.5/X*	4/916*
	1/750	1/915	1/1290
Mean	1.0 ^a /682	3.1 ^{a,b,A} /748 ^{A,B}	3.5 ^b /840 ^{A,B}
Treatment C, Cryoprotectant, no freeze	At flush	24 h	48h
	1/400	1/700	3/750
	1/700	1/750	1/1000
	1/325	1/830	1/1040
	1/1000	1/1330	1/1330
	2/650	2/1040*	2/1080*
Mean	1.2/615 ^a	1.2 ^B /930 ^{a,b,B}	1.3/1040 ^{b,B}
Treatment D, Culture control	At flush	24 h	48h
	1/400	1/800	1/830
	1/500	4/X*	5/X*
	1/500	1/790	1/916
	1/750	1/1080	1/1250
	1/1000	1/1250	3/915
Mean	1.0/630	1.6 ^{A,B} /980 ^B	2.2/977 ^{A,B}

An asterisk indicates an embryo that was ruptured. An X indicates a missing data point or an embryo that was damaged so that a measurement was unable to be made. Significant differences ($P < 0.05$) over time within groups are indicated with lower case letters. Significant differences between groups at specific time points are indicated with capitalized letters

Table 3

Diameters (μm) of pairs of embryos transferred to mares.

Treatment A Predehydration	Treatment B Control Freeze
400, 400*	400, 415*
415, 450	500, 500
540, 580	700 [†] , 700
700 [†] , 700	800, 800
700 [†] , 700	900, 950
700, 750	
1000 [†] , 1000	
1330 [†] , 1350	

An asterisk indicates the detection of pregnancy after transfer of the embryo pair. (Note: only one pregnancy in each mare was detected although two embryos were transferred).

[†] indicates an embryo that had a ruptured capsule upon thaw