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Effect of dehydration prior to cryopreservation of large equine embryos ☆

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ABSTRACT

Cryopreservation of equine embryos > 300 μm 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, day 7–8, grade 1, equine embryos 300–1350 μm 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 three-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 $\geqslant 3$ 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 μm embryos from treatment A, and the other one 400 and one 415 μm 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 μm in diameter (50–65% on average [19] and as high as 80% [18]); however, these protocols do not result in acceptable pregnancy rates for larger, more advanced equine embryos [11,18]. The value of cryopreserving equine embryos greater than 300 μm in diameter is linked to embryo recovery rates during uterine flushes. Equine embryos flushed from the mare 6 days after ovulation are generally smaller than 300 μm and have a higher survival rate after cryopreservation than more advanced embryos. However, the embryo recovery rate is higher when mares are flushed 7–8 days after ovulation, a stage at which the embryos are usually larger than 300 μm [19]. 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.

Attempts to freeze large equine embryos usually are fraught with low sample numbers, and most have resulted in unacceptable pregnancy rates. Maclellan et al. [10] 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 μm (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 damage to microfilaments and plasma membrane during freezing (43%, 3/7) [11]. However, other studies have found that pretreatment with cytochalasin B and trypsin can cause irreversible changes that complicate embryo handling and that likely are detrimental to embryo survival [22].

Young et al. [24] 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.

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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 two-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 day 15 pregnancy rate as glycerol (EG, 2/8; glycerol, 3/8) [6]. Pfaff [16] 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 with ethylene glycol. None of these characteristics was observed of embryos that had been frozen with 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 [13]. 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 to cryoprotectants of the capsule, an acellular glycoprotein coating secreted by the embryo beginning approximately 6 days after fertilization [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 [15], 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 stereomicrosccope, 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 μg, 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 days post-ovulation as previously described [19].

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 at the laboratory, embryos were rinsed through two drops (500 μl 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 1–5, 1 being excellent, 2 good, 3 fair, 4, poor, and 5 degenerate or dead [12]. Only embryos of grade 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 μm.

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% CO_2 in air at 38 °C. Twenty-six additional embryos were subjected to treatment 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: $CaCl_2$ concentration was reduced by half to 0.5 mM, glycine was increased from 0.1 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 develop-