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Cryopreservation of Day 8 equine embryos after blastocyst micromanipulation and vitrification

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ABSTRACT

Pregnancy rates after cryopreservation of large equine blastocyst stage embryos have remained lower than other domesticated livestock species. It is generally accepted that the embryonic capsule is the primary barrier to cryoprotectant entry into the embryo proper and techniques need to be developed to circumvent this obstacle. Therefore, the objective of this study was to develop an efficient Day 8 equine embryo cryopreservation protocol through blastocyst micromanipulation and vitrification. Grade 1 and 2 embryos recovered from mares (n = 15) 8 days after ovulation were used in these experiments. In experiment 1, the effect of either one- or two-puncture treatments before aspiration of blastocoel fluid and exposure to vitrification solutions was evaluated. No difference was detected in mean embryo volume across treatment groups after exposure to vitrification solutions or after 1, 24, 48, and 72 hours of culture. Percent of embryos re-expanding at 24 hours and percent of embryos showing diameter increase at 48 and 72 hours during *in vitro* culture were 100%, 83%, and 75% compared with 93%, 67%, and 50% for one- and two-puncture treatment groups, respectively. Capsule loss was 25% for one-puncture and 50% for two-puncture treatment groups. In experiment 2, no difference was detected in mean embryo volume for indirect introduction (aspiration of blastocoel fluid + equilibration) and direct introduction (injection of cryoprotectant into blastocoel cavity) treatment groups, after exposure to dilution solution or to culture medium. There was no difference in mean embryo volume for the indirect and direct introduction treatment groups after 1, 24, 48, and 72 hours of culture. Percent of embryos re-expanding at 24 hours and percent of embryos showing diameter increases at 48 and 72 hours during *in vitro* culture were 100%, 76.9%, and 69.2%, respectively, for both treatment groups. Those embryos subjected to the direct introduction treatment had a higher (P = 0.05) percent capsule loss (70%) compared with the indirect introduction treatment group (31%). The pregnancy rate after transfer of vitrified expanded Grade 1 blastocysts using the indirect introduction method was 83% (5/6). Three pregnancies were allowed to continue to term and resulted in the birth of three healthy foals. The vitrification protocol used in this study has the potential to become a key tool for the successful cryopreservation of equine expanded blastocysts.

1. Introduction

The cryopreservation of large equine embryos (≥300 μm) has typically resulted in pregnancy rates ranging from 0% to 38% [1–6]. The lack of the ability to successfully cryopreserve large equine blastocysts has been attributed to their relatively large blastocoel volume,

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increased size and cell number, intense mitotic activity, change in expression of membrane protein aquaporin, and development of the acellular glycoprotein capsule [7–13]. Most vitrification studies using large intact equine blastocysts have not resulted in acceptable pregnancy rates. Eldridge-Panuska et al. [3] evaluated the vitrification of expanded blastocysts (mean = 609 μm) using a vitrification protocol that has been used for the successful cryopreservation of equine embryos less than 300 μm, however, no pregnancies were reported in that study. Campos-Chillon et al. [14] used a four-step cryoprotectant addition protocol using ethylene glycol as the cryoprotectant for the vitrification of equine embryos and reported a 35% pregnancy rate in which pregnancies were from embryos that were between 300 and 400 μm in diameter.

The embryonic capsule develops by approximately Day 6.5, soon after the embryo enters the uterus coinciding with the onset of blastulation [15–17]. Although its functions have not been completely elucidated, it is essential for embryonic viability. The capsule appears to play an important role in embryo cell to cell interactions [18], and in addition to the mucin-like glycoproteins, the capsule contains proteins that may be involved in the transport of materials into and out of the developing embryo [19]. Because of the strong and elastic nature of the capsule, it has been proposed that it aids in physical protection of the embryo during the time it is subjected to constant myometrial contractions [20,21], which appear to facilitate maternal recognition during the embryonic mobility phase. This phase occurs between Day 7 and 16 and is required for the embryo to release an antiluteolytic signal, thus preventing CL regression [16]. The embryo mobility response is also thought to be facilitated by the antiadhesive properties of the capsule glycoproteins, which carry a high proportion of sialic acid residues. Loss of sialic acid residues from capsule glycoproteins would terminate the antiadhesion effect and is temporally associated with the fixation of the equine embryo on Day 17, indicating that it is a unique developmentally regulated mechanism of embryo mobility control [18].

The inverse relationship between cryoprotectant permeability and capsule thickness [8] leads us to believe the capsule is the primary barrier to successful cryopreservation of large expanded equine blastocysts [22,23]. Indeed, low permeability has been reported for both the cryoprotectants, glycerol and ethylene glycol, when used on capsulated equine embryos [11,24]. Several different approaches have been applied to overcome the low cryoprotectant permeability of the capsule before cryopreservation but have not resulted in acceptable pregnancy rates. Slow cooling cryopreservation after osmotic-induced dehydration of expanded blastocysts with a reduction of embryo volume by 45% resulted in an overall pregnancy rate of 33% for recipients receiving embryos less than 415 μm transferred in pairs [4]. Legrand et al. [25] reported a 75% pregnancy rate after enzymatic treatment and slow cooling cryopreservation of equine-expanded blastocysts (187–1581 μm), but attempts to replicate these results to date have not been successful [8,26]. Pretreatment of expanded blastocysts (300–1100 μm) with the microfilament inhibitor cytochalasin-B and slow cooling cryopreservation has resulted in similar pregnancy rates compared with control embryos (42% and 57%, respectively) [26]. The laser-assisted approach to vitrification has been evaluated resulting in a pregnancy rate of 44% at Days 12 to 14, but only one pregnancy remained viable to Day 23 [6]. Therefore, methods to introduce cryoprotectants without negatively impacting subsequent pregnancy rates are needed.

The ability to puncture and manipulate equine oocytes and embryos while still successfully producing offspring has allowed the application of assisted reproductive techniques such as intracytoplasmatic sperm injection, embryonic and somatic nuclear transfer, and preimplantation genetic diagnosis [27–29]. The success of these technologies demonstrates that capsule puncture may be a feasible alternative for the cryopreservation of large equine embryos.

In fact, breaching the embryonic capsule followed by extraction of blastocoel fluid has resulted in the successful vitrification of equine-expanded blastocysts with diameters ranging between 300 μm and 650 μm [7]. But to date, limited data exist on larger Day 8 equine embryos. Therefore, the objectives of this experiment were to determine if there were differences in *in vitro* re-expansion and capsule loss of Day 8 equine large expanded blastocysts subjected to vitrification solutions after one or two punctures and either an indirect or direct introduction of cryoprotectants. In addition, *in vivo* survival (by embryo transfer) was tested after indirect introduction of cryoprotectants and vitrification. Therefore, the objective of this study was to develop an efficient Day 8 equine embryo cryopreservation protocol through blastocyst micromanipulation and vitrification.

2. Materials and methods

These experiments were approved by the Louisiana State University Agricultural Center Institutional Animal Care and Use Committee.

2.1. In vivo embryo production

Fifteen quarter horse mares were used as embryo donors, and three stallions of known fertility were used as semen donors. The mares ranged in age from 5 to 16 years with body condition score ranging from 5 to 7 (9-point scale), and stallions were 4 and 12 years of age. Stallions were collected on the day of insemination, and all mares were subjected to uterine and ovarian ultrasonography daily using a 5-MHZ linear probe (Micromaxx, Sonosite Inc., Bothell, WA, USA). Mares that exhibited a follicle greater than or equal to 34 mm, uterine edema, and no CL were inseminated every other day until ovulation was detected (Day 0).

On Day 8 after ovulation, embryos were recovered nonsurgically as described by Scott et al. [24]. For embryo recovery, a Foley catheter (Agtech Inc., Manhatten, KS, USA) was introduced into the uterine body and the uterus lavaged with 2 L of prewarmed (37 °C) lactated ringer's (Hospira, Lake Forest, IL, USA) supplemented with 1% bovine calf serum (Hy Clone Inc., Logan, UT, USA). Embryos