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Vitrification of *in vitro*-produced and *in vivo*-recovered equine blastocysts in a clinical program

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ABSTRACT

There is a clinical demand for cryopreservation of both *in vivo*-recovered and *in vitro*-produced (IVP) equine embryos. We previously reported successful vitrification of expanded equine blastocysts in fine-diameter microloader pipette tips (MPTs) after blastocoel collapse, in a research setting. Here, we report the results of clinical application of the MPT vitrification technique for both *in vivo*-recovered and IVP blastocysts. *In vivo*-recovered blastocysts were obtained by referring veterinarians on Days 6 to 8 after ovulation, and shipped 1 to 10 hours to the laboratory before vitrification. IVP blastocysts (<300 μm in diameter) were produced by intracytoplasmic sperm injection and *in vitro* embryo culture. All vitrified-warmed embryos were shipped (0.5–12 hours) for transfer to recipient mares. In experiment 1, 47 IVP embryos from our clinical intracytoplasmic sperm injection program were vitrified using the MPT and transferred. The rates of initial pregnancy (59%) and foaling (45%) were equivalent to those for 52 IVP embryos from the same mare aspiration sessions and shipped for the same duration but transferred fresh (75% and 45%, respectively). The pregnancy and foaling rates for *in vivo*-recovered embryos were 76 and 71%, respectively for 17 small blastocysts (<300 μm in diameter), and 55 and 45%, respectively for 11 large blastocysts (303–608 μm in diameter, collapsed before vitrification; $P > 0.1$). In experiment 2, the MPT was cut lengthwise to form an open vitrification device, designated "Sujo". Research IVP blastocysts were vitrified at 1, 2, or 3 embryos per Sujo ($n = 34$ embryos), or singly on a commercial open device (Cryolock; $n = 11$). After warming, 97% and 91% of embryos, respectively, grew in culture. Similarly, culture of two *in vivo*-recovered large blastocysts after collapse and vitrification on Sujos both resulted in embryo growth. However, transfer of four *in vivo*-recovered expanded blastocysts after collapse, vitrification on Sujos, and warming resulted in only one foal. These data indicate that vitrification of equine IVP embryos and small *in vivo*-recovered embryos is efficient under clinical conditions. Collapse and vitrification of *in vivo*-recovered large blastocysts in MPT under our clinical conditions resulted in a 45% foaling rate. While numbers are low, use of an open vitrification system did not appear to improve results for these embryos.

1. Introduction

Cryopreservation of equine embryos allows embryos to be stored for transfer at desired times, and to be held while genetic tests are being performed. In addition, as the *in vitro* production (IVP) of equine embryos using intracytoplasmic sperm injection (ICSI) and embryo culture increases in efficiency, there is increasing demand from horse owners for embryo cryopreservation when embryos produced cannot be immediately transferred.

Cryopreservation of small (<300 μm in diameter) *in vivo*-recovered equine embryos, by either slow

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freezing or vitrification, is successful, achieving pregnancy rates after transfer of 45% to 67% [1,2]. However, cryopreservation of large embryos (>300 μm in diameter), such as those typically recovered for embryo transfer at Day 7 or 8 after ovulation, has been problematic (0%–38% pregnancy rates at Days 16–26; [1,3–5]). In an important development in this area, we reported up to a 71% normal pregnancy rate for expanded *in vivo*-recovered equine blastocysts (>300 μm in diameter) that were subjected to blastocoel collapse before vitrification and warming [6]. The embryos were vitrified using ethylene glycol and galactose as cryoprotectants; a fine-diameter microloader pipette tip (MPT) was used as the vitrification device.

To the best of our knowledge, there has been only one subsequent report on the use of blastocoel collapse for vitrification of equine embryos [7]. In that report, in a research setting, collapsed *in vivo*-recovered Day-8 equine blastocysts were vitrified using a commercial open device (Cryolock) using vitrification media previously successful with smaller equine embryos [1]. However, perhaps related to the system used to puncture the embryos, in the study of Diaz et al. [7], a large percentage (25%–70%, depending on treatment) of embryos underwent capsule loss, presumably rendering them nonviable. Overall, transfer of six intact vitrified-warmed blastocysts in that study resulted in five pregnancies (83%); three foals were produced from three pregnancies allowed to be maintained to term.

Little information is available on cryopreservation of IVP equine embryos. Galli et al. [8] reported a pregnancy rate of 9/13 (69%) after transfer of slow-frozen-thawed ICSI-IVP blastocysts, with 5/6 pregnant mares foaling at the time of that writing for a predicted 58% overall foaling rate. In a review paper, the same laboratory also reported a foaling rate of six foals + four ongoing pregnancies from 18 transfers (56%) of fresh and frozen-thawed embryos combined [9]. The methods for freezing and thawing were not detailed. To the best of our knowledge, these are the only publications available on foaling data after the transfer of cryopreserved ICSI-IVP blastocysts.

Vitrification has several advantages compared to slow freezing. It is faster and does not require programmable freezing equipment [10]. Vitrification avoids intracellular ice formation and thus potentially may result in higher embryo viability. Improved embryo survival has been reported for vitrification versus freezing in mouse, goats, and cattle [11–13], and vitrification resulted in improved perinatal outcome in humans and rabbits [14,15].

To better define the utility of equine embryo vitrification in clinical practice, more information about pregnancy and foaling rates after the transfer of vitrified-warmed equine embryos under a variety of systems is needed. Here, we report the foaling rate of 75 IVP, small *in vivo*-recovered, and large *in vivo*-recovered embryos that were vitrified, warmed, and transferred as part of a clinical program. We also report the results after vitrification of equine embryos using an open device produced by modifying the microloader tips.

2. Materials and methods

2.1. Experiment 1: vitrification of IVP and in vivo-recovered blastocysts in microloader tips in a clinical program

2.1.1. IVP embryos

The IVP embryos used in this study were produced by ICSI of *in vitro*-matured oocytes from client-owned mares over a 3-year period. Oocytes were recovered from immature follicles by transvaginal ultrasound-guided aspiration [16] either at the Veterinary Medical Teaching Hospital at Texas A&M University or at referring veterinary practices, as part of the Texas A&M Clinical Equine ICSI Program.

Immature oocytes recovered as Texas A&M were identified under a dissection microscope, then held in Earle's/Hanks' holding medium (40% M199 with Earle's salts (Invitrogen), 40% M199 with Hanks' salts, and 20% fetal bovine serum (FBS); [17]) overnight. Oocytes recovered by referring veterinarians were shipped overnight or by courier to the laboratory in either Earle's/Hanks' medium or in commercial embryo holding medium. The morning after oocyte recovery, the oocytes were placed in maturation medium (M199 with Earle's salts with 10% FBS and 5 mU/mL FSH [Sioux Biochemicals, Sioux Center, IA, USA]) and cultured for 30 hours as previously described [18]. Oocytes from one mare in this series were directly matured without overnight holding; two embryos were produced from these oocytes, resulting in one foal.

Mature oocytes were fertilized by ICSI and cultured *in vitro* in a commercial human embryo culture medium (GB; Global medium, LifeGlobal, Guilford, CT, USA) with 10% FBS and added glucose, as described previously [19]. Embryos developing to the blastocyst stage between Days 7 and 9 of culture were included in this analysis.

2.1.2. In vivo-recovered embryos

In vivo-recovered embryos were recovered by referring veterinarians by uterine flush of inseminated mares on Days 6 to 8 after ovulation, over a 6-year period. The embryos were shipped to the laboratory by same-day courier or by air. Embryos were classified as to stage of development and diameter. All *in vivo*-recovered embryos greater than 300 μm in diameter were subjected to micromanipulation to remove 70% or more of blastocoel fluid, with the micropipette placed at the periphery of the trophoblast, as previously described [6]. The embryos were then vitrified as described below, using the MPT technique.

2.1.3. Vitrification

Embryos were vitrified and warmed as described previously [6] using the EG/s method, that is, vitrified in medium containing ethylene glycol and galactose, and warmed in medium containing sucrose. Briefly, embryos were held in DMEM/F-12 with 20% FBS (DMEM/F-12/FBS) for a variable period, then transferred into 1.5 M ethylene glycol in DMEM/F-12/FBS for 5 minutes, and then moved into 7 M ethylene glycol and 0.6 M galactose in DMEM/F-12/FBS. Within 1 minute, embryos were loaded with a minimum amount of fluid into an MPT (Eppendorf,