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Blastocyst development after intracytoplasmic sperm injection of equine oocytes vitrified at the germinal-vesicle stage

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ABSTRACT

We evaluated the meiotic and developmental competence of GV-stage equine oocytes vitrified under different conditions. In a preliminary study, using dimethyl sulfoxide (D), ethylene glycol (EG) and sucrose (S) as cryoprotectants, the maturation rate was higher for cumulus-oocyte complexes (COCs) held overnight before vitrification (37%) than for those vitrified immediately (14%; $P < 0.05$). Thereafter, all COCs were held overnight before vitrification. In Experiment 1 we compared 1 min (1m) and 4 min (4m) exposure to vitrification and warming solutions; oocytes that subsequently matured were fertilized by ICSI. The maturation rate was similar between timing groups (29–36%), but was significantly lower than that for controls (73%). The 1m treatment yielded one blastocyst (11%), vs. 19% in controls. In Experiment 2, propylene glycol (PG) and trehalose (T) were also used. We compared two base solutions: M199 with 10% FBS (M199+), and 100% FBS; three cryoprotectant combinations: D-EG-S; PG-EG-S; and PG-EG-T; and two timings in vitrification solution: ~30 s (30s) and 1 min (1m). The most effective treatment (FBS/PG-EG-T/30s) yielded 42% maturation, 80% cleavage and 1 blastocyst (10%), vs. 49%, 93% and 29%, respectively for controls ($P > 0.1$). In Experiment 3, we evaluated the toxicity of the M199/D-EG-S/1m and FBS/PG-EG-T/30s treatments, without actual vitrification. These treatments did not affect maturation but both significantly reduced blastocyst development (0% and 0%, vs. 21% for controls). This represents the second report of blastocyst development after vitrification of GV-stage equine oocytes, and presents the highest developmental competence yet achieved; however, more work is needed to increase the efficiency of this system.

1. Introduction

Oocyte cryopreservation has the potential to preserve female genetics. Establishing methods for equine oocyte vitrification would be a great benefit to the horse industry, for both clinical and research use. Clinically, vitrification would be applicable in cases in which oocytes are recovered from mares post-mortem, or prophylactically as mares age, and owners wish to delay the decision on which sire to use until a later date. Additionally, oocytes from valuable mares could be recovered and vitrified in locations in which assisted reproductive techniques are not well-developed, and then shipped to an assisted reproduction laboratory for embryo production. In some countries, the availability of equine oocytes for research is greatly limited – as in the United States in which all horse slaughterhouses were closed in 2007– thus oocyte cryopreservation and transport could also offer a tool to provide a supply of equine oocytes for study.

Cryopreservation of germinal-vesicle stage (GV) oocytes would allow collection and preservation of oocytes at a remote location, without the requirement for culture facilities. Cryopreservation of GV-stage oocytes is generally associated with lower survival after vitrification than that for metaphase II (MII) oocytes [16,37], associated with a higher sensitivity of GV-stage oocytes to osmotic stress [2]. Nevertheless, developmental competence of those oocytes that survive vitrification may be higher for GV-stage than for MII oocytes [37], and embryo production after vitrification of GV-

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stage oocytes has been described in several species [1,4,39,42].

In humans, efficient vitrification systems have been developed for mature oocytes [12,27]. Using similar techniques for vitrification of mature (metaphase II) equine oocytes, moderate survival [25] and pregnancy rates [28] have been obtained. Vitrification of GV-stage equine oocytes has also been described, with variable rates of maturation in vitro after warming [13,14,24,25,36,40]. However, to the best of our knowledge, only one blastocyst has been reported from equine oocytes that were vitrified at the GV stage; this blastocyst was produced after vitrification of 382 oocytes, and intracytoplasmic sperm injection (ICSI) of 156 oocytes that reached MII after warming and in vitro maturation [40]. These authors reported minimal toxicity of vitrification media containing dimethyl sulfoxide (D), ethylene glycol (EG), and sucrose (S) as cryoprotectants. After exposure to these media without vitrification, equine GV-stage oocytes yielded a 50–65% maturation rate and 8–9% blastocyst rate, compared to 53–56% and 10–17%, respectively, for controls. When vitrification was performed, this system supported a maturation rate similar to or slightly lower than that for controls (42–54%). However the blastocyst rate for vitrified-warmed oocytes was extremely low (<1%), suggesting that the main detrimental effect of vitrification was chilling injury [40].

More recently, Rosati et al. [36] reported an in vitro maturation (IVM) rate equivalent to that for controls (61%) after vitrification of GV-stage equine oocytes, using increasing concentrations of D-EG over 14 min, then sucrose as non-permeable cryoprotectant, a technique similar to that used successfully for vitrification of mature human oocytes [12]. However, Rosati et al. [36] did not assess embryo development. The high maturation rate achieved using this technique, compared with other reports on equine GV-stage oocyte vitrification, warrants investigation of whether this method also can support developmental competence after fertilization.

Porcine oocytes and embryos are sensitive to cryopreservation, attributed to the large amount of lipid in the cytoplasm [20,32,33]. Somfai et al. [38] showed that the combination of propylene glycol (PG) and EG provided greater embryo development after vitrification of GV-stage porcine oocytes than did either CPA alone. The same research group obtained the first piglets after vitrification of GV-stage oocytes, using this combined CPA treatment [39]. Equine oocytes also have a large amount of cytoplasmic lipid [19]. To the best of our knowledge, the effect of combination of different CPAs on viability and developmental competence after vitrification of equine GV-stage oocytes has not yet been studied.

Slow freezing and vitrification of somatic cells, such as mammalian stem cells, may be performed using 100% serum as a base medium [18,35]. Cryopreservation of gonadal tissues and semen was improved by addition of fetal bovine serum (FBS) to the freezing medium [7,15,29], and a high concentration of FBS (80%) in combination with 10% DMSO was efficient in cryopreserving germ and germ-stem cells in buffalo testis [15]. Serum has higher viscosity than does standard medium, and increased viscosity is associated with a higher likelihood of a solution achieving the vitrified state when cooled [43]. These studies suggest that the use of a high concentration of FBS as base medium may offer some protectant qualities during cryopreservation; however, the use of FBS as a base medium in oocyte vitrification remains unexplored.

Vitrification devices commonly limit the number of oocytes to be vitrified at one time, which would limit the usefulness of vitrification as a means to collect large numbers of oocytes for transport to the laboratory. Abe et al. [1] used a nylon-mesh vitrification device that allows preservation of a large number of oocytes simultaneously, and as an open method, allows a high cooling rate. Such a method would increase the efficiency of vitrification as a large-scale method for collecting equine oocytes for research.

The purpose of this study was to evaluate the meiotic and developmental competence of GV-stage equine oocytes vitrified under different conditions, with the goal of developing an effective vitrification technique. Based on previous studies on equine, porcine and bovine GV-stage oocytes [1,36,38,39], we used steel mesh as a vitrification device and tested different media and cryoprotective agents, and different timing during the vitrification and warming processes. We also evaluated the toxicity of the vitrification systems developed.

2. Materials and methods

2.1. Experimental animals and materials

Fifteen Quarter Horse-type mares weighing 500–600 kg and aged 4–16 years were used as oocyte donors. The mares were housed outside in paddocks and were fed hay and water *ad libitum*. All experimental procedures were performed according to the *United States Government and Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research and Training* and were approved by Laboratory Animal Care Committee at Texas A&M University.

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and media from Invitrogen (Carlsbad, CA, USA) unless otherwise specified.

2.2. Recovery of COCs via transvaginal ultrasound-guided follicular aspiration

When oocytes were obtained by transvaginal ultrasound-guided follicle aspiration (TVA), all follicles $\geq$ 5 mm in diameter were aspirated by TVA in each mare approximately once every two weeks. There was no attempt to manipulate or stage the cycle between aspiration sessions. The TVA procedure and isolation of cumulus-oocyte complexes (COCs) were conducted as previously described [26]. Follicles >30 mm diameter were aspirated separately, to avoid including expanded granulosa or oocytes from preovulatory follicles in the immature-follicle aspirate. Aspirates from follicles over 30 mm were sampled; if granulosa cells were compact the aspirated fluid was then combined with that from the smaller follicles; if the granulosa was expanded, these aspirates were not used in this project. The aspirated fluid was filtered through an embryo filter (EmCon filter, Immuno Systems, Inc., Spring Valley, WI, USA) and the COCs were recovered from the collected cellular material. As they were located, the COCs were placed in a 35-mm polystyrene Petri dish (Falcon, Corning Incorporated, NY, USA) containing M199 with Hank's salts and 25 mM HEPES, supplemented with 10% FBS.

2.3. Recovery of COCs from slaughterhouse-derived ovaries

Ovaries were obtained at a slaughterhouse (Canada), placed in an insulated container and transported to the laboratory at the University of Montreal at room temperature (~22 °C; 30–45 min transport time). All visible follicles <30 mm diameter on the ovary surface were opened with a scalpel blade and the granulosa layer was aspirated using a 14-ga needle connected to a vacuum pump (Gast, IDEX Corporation, Benton Harbor, US) set to aspirate approximately 20 mL of fluid per minute. The aspiration apparatus and tubing was rinsed between follicles with M199 with Hank's salts and 25 mM HEPES supplemented with 0.4% FBS, 8 IU/mL heparin (#H3149) and 25 μg/mL gentamicin. The aspirated fluid was collected into 250-mL sterilized plastic bottles. After all follicles visible on the surface had been processed, the ovaries were cut to locate the follicles within the ovarian stroma and the granulosa