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Original Research

Influence of Embryonic Size and Manipulation on Pregnancy Rates of Mares After Transfer of Cryopreserved Equine Embryos

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ABSTRACT

Many years of poor results of equine embryo cryopreservation has produced a lack of confidence in this technique. Embryo cryopreservation has been successfully used for more than 20 years in other species like bovine and human. The large size of the embryos and the presence of a capsule impermeable to cryoprotectants have been the two main reasons for the failure. In the last few years, a mayor breakthrough for this technique was obtained when large equine embryos could be successfully cryopreserved after breaching the capsule and collapsing the blastocoel cavity. In the present study, we compared the pregnancy rates obtained by vitrification or cryopreservation by slow freezing of embryos smaller than 300 μm. No difference was found between vitrification and slow freezing of embryos <180 μm (pregnancy rate on day 16: 34/61, 55.7%; 6/8, 75%) but produced very low results for embryos between 180 and 300 μm in diameter (0/11, 0%; 1/7, 14.3%). Embryos larger than 300 μm were collapsed before cryopreservation, and two different types of carriers, hemi-straw or Stripper-Tip, were used for vitrification. High pregnancy rates were obtained when the hemi-straw was used as a carrier (7/10, 70% vs. 0/5, 0%), demonstrating that a minimum vitrification volume was essential to preserve the embryo viability. These findings establish that, due to the large range in diameter, equine embryos need to be cryopreserved using different protocols depending on their size.

1. Introduction

Since cryopreservation of equine embryos was first described, size seemed to have an obvious effect on the success rate of cryopreservation [1]. Until now, cryopreservation is not routinely used due to this problem. Only low pregnancy rates can be achieved for intact embryos larger than 300 μm. These embryos are present in the uterus around day 7 postovulation. On one hand, efficiency of cryopreservation can be improved when embryos are collected at the morula to early blastocyst stage (days 6–6.5 postovulation), resulting in an acceptable pregnancy rate (55%–70%) with both conventional cryopreservation and vitrification [2–4]. On the other hand, the recovery rate at this time is low [5] and difficult to schedule in practice.

The reasons for the low success rates of cryopreserving embryos >300 μm in diameter seem to be multifactorial

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[6]. Among these are the large embryonic size and blastocoel volume [7], rapid increase in cell number and therefore high mitotic activity [8,9], the presence of an embryo capsule [8,10] and cryopreservation techniques not fulfilling specific demands of expanded blastocysts [6].

A major step regarding successful cryopreservation of embryos >300 μm in diameter was achieved by Choi et al [7], who collapsed the blastocoele cavity prior to vitrification. This was done by the puncture of the blastocyst and aspiration of the blastocoele fluid. Pregnancy rates of embryos >300 μm in diameter following these manipulations and vitrification revealed to be similar for that of embryos <300 μm (71%, 5 of 7). The authors concluded that the decrease in volume seems to be the most important factor for successful cryopreservation, as a correlation between extent of fluid aspiration and higher pregnancy rates was observed.

Some authors suggest that conventional or slow freezing might be more compatible to embryonic survival [11–13]. In their report, Hendriks et al [13] studied the survival of intact equine embryos after vitrification or slow freezing and they concluded that vitrification of large embryos caused a higher incidence of dead cells than slow freezing. Recently, Diaz et al [14] reported high pregnancy rates (83.3%) and birth of healthy foals after transfer of six collapsed, vitrified, and warmed day 8 large (821–1,168 μm) equine embryos.

A reliable cryopreservation management for equine embryos has a great potential, as it would allow an easier management of recipient mares (no synchronization required) and international shipping.

Therefore, the aim of our work was to define the optimal circumstances for cryopreservation of equine embryos of different sizes.

2. Materials and Methods

2.1. Animals

All mares used for the present research project were sport horses between 2 and 18 years old and belonged to a private breeding farm. Recipient mares were healthy, with body size similar to that of the donor and with or without a foal.

2.2. Management of the Donor Mares

Donors were inseminated with chilled or frozen semen, depending on availability. All the stallions included in this study had proven, but varying fertility. If insemination was done with chilled semen, mares were inseminated with 50 to 200 million spermatozoa per insemination. When frozen semen was used, mares were inseminated with one or two straws (50–200 sperm/straw).

Mares received 2,500 IU of human chorionic gonadotropin (hCG) i.v. when a follicle of at least 35 mm and uterine edema was detected. If chilled semen was used, mares were inseminated 24 hours post-hCG. When frozen semen was used, mares were monitored 10 and 18 hours post-hCG, then every 6 hours until ovulation occurred (day 0) and then inseminated.

All mares were inseminated with chilled or frozen semen by deep intracornual insemination using a deep intrauterine insemination pipette for 0.5-mL french straws (Minitüb, Germany).

2.3. Embryo Recovery

Mares were flushed between days 6 and 8 postovulation in order to collect embryos of different sizes. Uterine lavage was done with 2 L of Ringer lactate solution (B. Braun Melsungen AG, Germany) through a Foley catheter connected to a two-way flushing/collection system leading the fluid through an EmCon embryo filter (Agtech Inc). After flushing, fluid was immediately searched for embryos under a stereo-microscope. The quality of the embryos was registered, and only grade 1 embryos were used in this study [15]. Grade 1 embryos were measured using a calibrated micrometer eyepiece and assigned to three different groups according to their diameter: small embryos (<180 μm), medium-sized embryos (180–300 μm), and large embryos (>300 μm). As a control group, some of the embryos were cooled to 5°C in an Equitainer and transported between 6 and 10 hours and then transferred intact.

2.4. Assisted Blastocoel Collapse

Assisted blastocoel collapse was done directly after recovery and classification, only on embryos larger than 300 μm. The procedure was performed as described previously [16] and accomplished by placing the embryos in 50-μL microdroplets of Dulbecco-modified phosphate-buffered saline without calcium and magnesium supplemented with 10% fetal bovine serum (FBS) and 50 μg/mL of gentamicin under mineral oil, on an inverted microscope (Olympus IX73, Olympus, Germany) equipped with a micromanipulation system (InjectMan, Eppendorff, Germany). Embryos were held in place by suction of a holding pipette, and the inner cell mass was placed 90° clockwise away from the holding pipette (MPH-XLG, ORIGIO, Humagen Pipets). Then, the embryo capsule was punctured with a beveled micropipette (9 μm ID, ORIGIO, Humagen Pipets), and all the blastocoele fluid was aspirated until the embryo was completely collapsed, as shown in Fig. 1.

2.5. Embryo Cryopreservation and Warming

For vitrification, two different methods were used for embryos smaller or larger than 300 μm. For embryos < 300 μm, a commercial equine vitrification kit (Bioniche Animal Health, Bogart, GA) was used according to the manufacturer's instructions.

Briefly, embryos were incubated in VS1 for 5 minutes, in VS2 for 5 minutes, and then in VS3. The embryos were individually loaded into a 0.25-mL straw as shown in Fig. 2, and within 45 to 60 seconds of having placed the embryo in VS3, the straw was labeled and sealed. Then, the straw was left in liquid nitrogen vapor for 1 minute and finally plunged into liquid nitrogen for storage until warming. For warming, the straw was removed from the liquid nitrogen and held at room temperature air for 5 to 10 seconds, then placed in a water bath at 35°C for 20 seconds. Holding the