Manual collapse of blastocoels in not effective in increasing the viability of vitrified equine embryos

Colabamento manual da blastocele não foi efetivo para aumentar a viabilidade de embriões equinos vitrificados

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Received: 10/03/2021. Accepted: 27/09/2021

ABSTRACT: Embryo cryopreservation methods have been used for commercialization and formation of genetic banks. Cryopreservation of equine embryos ≤300 μm in diameter, collected at days 6-6.5 after ovulation, allows satisfactory pregnancy rates. However, higher embryo collection rates in mares are obtained when uterine flush is performed between days 7 and 8 after ovulation when embryos are >300 μm in diameter, needing blastocoel collapse for satisfactory resistance to cryopreservation by vitrification. To evaluate the viability of simplified blastocoel collapse by embryo puncture with low technology and low-cost equipment, 22 embryos, collected at day 8 post-ovulation (D8), were allocated to the following groups: (1) micropuncture with a 30 G needle, assisted by a mechanical micromanipulator, before vitrification (n=4); (2) manual blade microsection before vitrification (n=6); (3) no manipulation prior to vitrification (n=8); and (4) freshly inovulated embryos (n=4). Despite the high re-expansion rates observed after vitrification, embryos manipulated prior to vitrification (groups MP and MS) did not result in pregnancy 25 days after transfer. On the other hand, embryos from groups NM (non-micromanipulated) and FR (freshly inovulated) resulted in pregnancies at 25 days. Under the conditions of the present study, manual blastocoel collapse was not efficient in increasing cryotolerance to vitrification among large embryos, requiring improvements to obtain pregnancies.

KEYWORDS: cryopreservation; reproductive biotechnology; blastocyst; embryo micromanipulation.

INTRODUCTION

Flushing, recovery and inovulation of non-cryopreserved (fresh) equine embryos, has been consolidated and used since the 1970s (Allen e Rowson, 1975). Embryo collection usually takes place between the 7th and 8th day after ovulation, when embryo diameter varies from 130 to 1344 μm (McCue et al., 2010; Cuervo-Arango et al., 2019). Satisfactory pregnancy rates with cryopreserved equine embryos are obtained with embryos with diameter lower than 300 μm in, i.e., compact morula or early blastocyst (Slade et al., 1985; Eldridge-Panuska et al., 2005), which requires uterine flush to be performed nearly 6.5 days after ovulation (McCue et al., 2010),...
before the capsule is completely formed. However, during this period, embryos are collected in less than 50% of flushings. The low recovery rate is related to the possible delay in the displacement of the embryo through the oviduct (Hinrichs et al., 1995). Therefore, a longer interval between ovulation and collection is related to an increase in the embryo recovery rate (Battut et al., 1997) and, consequently, to the recovery of embryos larger than 300 μm.

Although several studies have aimed to improve cryopreservation techniques for equine embryos, alternatives for cryopreservation of larger structures (>300 μm) still have limitations (Barfield et al., 2009; Choi et al., 2010; Scherzer et al., 2011; Diaz et al., 2016; Ferris et al., 2016; Weiss et al., 2016; Sanchez et al., 2017). Cryopreservation methods lead to a rearrangement of the biological membrane structure, changing its functionality (Holt, 2000). Among the cryopreservation techniques, vitrification is an alternative to slow freezing, as it is a process that prevents the formation of ice crystals, resulting in an amorphous stage with an appearance similar to glass. To achieve this condition, it is necessary to reduce the volume of the samples, to increase the concentrations of cryoprotectants and to use support systems that allow high speed cooling (Vajta e Nagy, 2006). Equine embryos larger than 300 μm, usually recovered from D7 onwards, where the embryo recovery rate is greater (Battut et al., 1997), have structural characteristics which impair the cryopreservation process. To reduce the embryonic volume, several techniques are being tested (reviewed by Bohm et al., 2020), such as osmotic dehydration (Barfield et al., 2009); laser microporferation (Scherzer et al., 2011); blastocoel manual micropuncture (Ferris et al., 2016) or micromanipulation assistance (Choi et al., 2010; Diaz et al., 2016; Weiss et al., 2016; Sanchez et al., 2017). Currently, these techniques are normally executed with micromanipulators, which are used mainly in research centers due to their high cost and the need for specialized training for operation (Bredbacka et al., 1995), so they have limited use in commercial field practice. At the same time, there has been an improvement in the embryo survival rate after vitrification, through improvements in the vitrification solution, for example, with the addition of dimethyl sulfoxide (DMSO) and the use of open supports (Weiss et al., 2016; Sanchez et al., 2017; Wilsher et al., 2018).

According to the available information, the association of blastocoel collapse by micromanipulation with vitrification in open supports, and the use of an adequate vitrification solution, represent promising methods for the cryopreservation of equine embryos with a diameter > 300 μm. However, the use of expensive equipment limits their applicability. To address this problem, this study proposes the use of the manual micromanipulation technique developed for performing embryonic biopsies under field conditions (Bredbacka et al., 1995) as an alternative for collapsing the blastocoel of equine embryos prior to vitrification.

MATERIAL AND METHODS
All described procedures were approved by the Animal Experimentation Ethics Committee from Federal University of Pelotas (Document 110/2018/CEEA/REITORIA).

Twenty embryo-donor mares, from 2 to 19 years old, predominantly from the Crioulo breed, were used. Thirty-five embryos were collected from 65 uterine flushes. Previously, the donor mares were submitted to ultrasonographic control of follicular development, to identify the appropriate time for ovulation induction. When the follicle reached 35mm, ovulation was induced with 1000 IU hCG (Human Chorionic Gonadotropin) (Jacob et al., 2012). Artificial inseminations were conducted with fresh or refrigerated semen, with at least 500 x 10⁶ motile spermatozoa, 24 hours after ovulation induction (Jacob et al., 2012). Ovulation time was considered as day 0 (D0) and all uterine flushes were performed on D8 (McCue e Squires, 2015).

The closed system technique (Scott et al., 2012) was used for uterine flushing, consuming, on average, 3 liters of ringer lactate per procedure. The contents of the embryo collecting filter were transferred to a Petri dish, allowing the location and measurement of the embryo under a stereomicroscope. Embryo diameter was determined by a graduated ocular lens. After these procedures, the embryos were washed in appropriate media and kept in a holding medium – HM [medium 199 with hepes + 10% bovine calf serum (BCS)] (McCue e Squires, 2015), on a warming table at 37°C, until the allocation to the experimental groups.

Embryos were allocated to four groups: MP - micropuncture with a 30 G needle assisted by a mechanical micromanipulator before vitrification (n=4); MS - manual microsection by blade before vitrification (n=6); NM - no manipulation prior to vitrification (n=8); and FR – control freshly inovulated (n=4).

All vitrification procedures were carried out in handmade hemi-straws (Vanderzwalmen et al., 2000). Initially, embryos were exposed to stabilization solution – SS [7.5% Dimethyl sulfoxide (DMSO) + 7.5% Ethylene Glycol (EG) in HM] at 37 °C, for 150 seconds. Then, the embryos were transferred to the vitrification solution - VS [15% DMSO + 15% EG + 0.5M Sucrose (SUC) + 20% BCS in medium 199]. Each embryo, with 2 μL of VS, was placed in a hemi-straw and immediately immersed in liquid nitrogen for 30 seconds (Scherzer et al., 2011). Each hemi-straw containing an embryo was placed in a 0.5 mL straw previously identified for storage in a cryogenic cylinder until warming.

Warming was performed as described by (Scherzer et al., 2007), with modifications. Hemi-straws containing embryos were exposed to air for 10 seconds and then immersed in 39°C warming solutions (WS), using a stepwise method: 30 seconds in WS1 [0.5M SUC in HM], and 4 minutes in WS2 (0.25M SUC in HM). After this, warmed embryos were transferred to HM for embryo loading in a 0.5 mL straw for inovation (McCue e Squires, 2015).
In the MP group, embryos were punctured with a 30G needle (Ferris et al., 2016), using mechanical micromanipulator assistance. After fixing the embryo in a holding pipette, the needle was inserted into the blastocoel from the opposite side of the embryonic mass under stereomicroscope. In this group, the average diameter of the embryos was 900 μm (660-1500 μm).

For the MS group, the embryos were submitted to manual microsection methodology (Bredbacka et al., 1995), using a razor-blade fragment. An incision was made in the blastocoel, on the opposite side of the embryonic mass. In this group, the average diameter of the embryos was 940 μm (660-1500 μm).

In the NM group, the embryos were not micromanipulated before vitrification. In this group, embryos had an average of 528 μm (180-1140 μm). Embryos in the FR group were not micromanipulated and not vitrified before inovulation. In this group, embryos had an average 360 μm (180-480 μm).

Recipient mares used for embryo inovulation were predominantly from the Crioulo breed, with an average age of 5.2 years (between 4 and 14 years), kept in native pasture, with mineral salt supplementation and ad libitum access to water. The recipients were kept separate from the donors, and their estrous cycle was monitored by ultrasound-assisted rectal palpation. The day of ovulation was considered as day 0 (D0), and all embryos were inovulated when the recipients were at D5 of the cycle, that is, 3 days earlier than the donor (Jacob et al., 2012).

Blastocoel collapse of embryos from the MP or MS groups was confirmed by visualizing the contraction of the structure and consequent reduction of the embryonic diameter under a stereomicroscope. Before inovulation, all embryos were evaluated according to their re-expansion capacity in HM after warming.

For inovulation, embryos were loaded in sterile 0.5 ml straws in the center of 3 columns of the holding medium, separated by air bubbles. The straw was placed in an embryo inovulator and in an inovulation sheath protected by a sterile sanitary jacket (Mccue e Squires, 2015).

After inovulation, the recipients were kept in the same housing conditions, and the first pregnancy diagnosis was made eight days after the procedure. The first diagnosis was made by ultrasonography, verifying the presence of the embryonic vesicle (Mccue e Squires, 2015), and the confirmation was made seven days later (Wilsher, S. et al., 2020).

Recipient mares had the following data recorded: 1) day of ovulation; 2) day of embryo inovulation in relation to ovulation; 3) first pregnancy diagnosis 8 days after inovulation; positive (presence of embryonic vesicle) or negative (absence of embryonic vesicle); 4) second pregnancy diagnosis 7 days after the first one.

After verification of normality by the Shapiro-Wilk test, the size of the embryos was compared by ANOVA and the means contrasted by the Tukey test (significance level of P < 0.05). The other data were not submitted to statistical analysis due to the low number of samples.

RESULTS AND DISCUSSION

The embryo recovery rate was 53% (35/66), which is similar to previous descriptions in the literature (Mccue et al., 2010; Jacob et al., 2012; Mccue e Squires, 2015). The average embryo diameter (676.9 μm) and its variation (from 180 to 1560 μm) was also similar to those described elsewhere (Mccue et al., 2010; Scherzer et al., 2011; Mccue e Squires, 2015; Ferris et al., 2016; Wilsher, Sandra et al., 2020). From all embryos obtained, 85.7% (30/35) were at the expanded blastocyst stage, having similar quality to those in previous studies (Mccue et al., 2010; Mccue e Squires, 2015; Ferris et al., 2016; Cuervo-Arango et al., 2018). These data indicate that the embryos obtained in this experiment are similar to those evaluated in previous studies on the subject (Choi et al., 2011; Scherzer et al., 2011; Wilsher, S. et al., 2020; Wilsher, Sandra et al., 2020).

Manual collapse of the blastocoel was not effective in increasing the pregnancy rate with vitrified equine embryos (Table 1). The pregnancy rate for the FR group was consistent with the rates described in the literature (Jacob et al., 2012; Cuervo-Arango et al., 2018), evidencing the technical capacity of the veterinarian who performed the procedures. Embryos with a larger diameter were selected to undergo the

Table 1. Embryo diameter prior to handling, and pregnancy rates 16 and 25 days after inovulation in recipient mares.

| Experimental group | MP         | MS         | NM         | FR         |
|--------------------|------------|------------|------------|------------|
| Embryo diameter (μm) | 900 ±1304±| 940 ±106.5| 502.5 ±92.2| 390 ±1304±|
| Amplitude          | 660-1560   | 660-1500   | 180-140    | 180-480    |
| Pregnancy rate (16 days) | 1/4 (25%)  | 2/6 (33.7%)| 4/8 (50%)  | 3/4 (75%)  |
| Pregnancy rate (25 days) | 0/4 (0%)   | 0/6 (0%)   | 1/8 (12.5%)| 3/4 (75%)  |
| Embryo re-expansion rate | 3/4 (75%)  | 4/6 (66.7%)| 6/8 (75%)  | -          |

MP: vitrification after micropuncture with a 30 G needle assisted by a micromanipulator; MS: vitrification after manual microsection by blade with manual control; NM: vitrification without manipulation; FR: inovulation without micromanipulation and vitrification. ±Mean ± SEM with distinct exponents differ by P < 0.05.
The collapse of the blastocoel from large embryos and subsequent vitrification proved to be promising in studies that used micro-precision equipment, keeping most of the embryonic capsule viable (Choi et al., 2010; Scherzer et al., 2011; Diaz et al., 2016; Weiss et al., 2016; Sanchez et al., 2017; Wilsher et al., 2018). However, when a greater rupture of the embryonic capsule is promoted, as probably occurred in the present study, the pregnancy rates are negatively affected (Wilsher, S. et al., 2020). Although we have not evaluated the embryonic capsule, (Stout et al., 2005) and (Wilsher, Sandra et al., 2020) showed the essential role of this structure for embryonic survival in the early stages of pregnancy. Thus, apparently, the manipulation techniques used in the present study caused severe lesions in the capsule, preventing embryonic development after inovulation. Confirmation of the extent of the capsule lesion could be evaluated as described by (Skidmore et al., 1989), although we chose to inovulate the embryos and assess the pregnancy rate.

Embryos from the MP group, which were punctured using a 30G needle attached to a micromanipulator, generated only one pregnancy (25%; 1/4), diagnosed at 16 days of gestation, but not confirmed at 25 days. These results disagree with those described by (Ferris et al., 2016), who reported 46% pregnancy rate after 14 days. It is noteworthy that in that study, a needle with smaller diameter should have caused less damage to the embryo, resulting in a higher pregnancy rate.

CONCLUSION

The technique of manual collapse of the blastocoel induced by a needle and by a blade was not efficient in sustaining embryo viability after the reduction of the embryonic diameter for vitrification purposes.

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