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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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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.

RESUMO: Métodos de criopreservação de embriões têm sido utilizados com diversos objetivos. Maiores taxas de coleta embrionária em éguas com lavagem uterina realizada 7 a 8 dias pós ovulação. A criopreservação de embriões equinos com diâmetro <300 µm (6-6,5 dias após a ovulação) permite a obtenção de taxas de prenhez satisfatórias. Embriões com diâmetro >300 µm (7º dia pós-ovulação) somente são adequadamente criopreservados quando submetidos a colabamento da blastocele. Objetivando avaliar a viabilidade da punção da blastocele com equipamento de baixa sofisticação e custo, 22 embriões coletados no 8º. dia pós-ovulação (D8) foram alocados aos seguintes grupos: (1) micropunção com uma agulha 30 G assistida por micromanipulador antes da vitrificação (n=4); (2) microssecção manual por lâmina antes da vitrificação (n=6); (3) sem manipulação anterior à vitrificação (n=8); e (4) transferidos a fresco (n=4). Apesar de altas taxas de reexpansão após a criopreservação, os embriões manipulados previamente a vitrificação não resultaram em prenhez aos 25 dias. Tanto os embriões não micromanipulados, quanto os transferidos a fresco resultaram em prenhezes aos 25 dias. A microssecção manual não se mostrou eficiente como método para aumento da criotolerância de embriões grandes, necessitando um aprimoramento visando a obtenção de prenhezes.

PALAVRAS-CHAVE: criopreservação; biotecnologia da reprodução; blastocisto; micromanipulação embrionária.

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),

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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 e Choi, 2016). 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 (Bohn *et al.*, 2020), such as osmotic dehydration (Barfield *et al.*, 2009); laser microperforation (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 IM hCG (Human Chorionic Gonadotropin)(Jacob *et al.*, 2012). Artificial inseminations were conducted with fresh or refrigerated semen, with at least 500 x 10^6 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 inovulation (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 (660-1560 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 a 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)				
Average	900 ± 130.4[ab]	940 ± 106.5[a]	502.5 ± 92.2[b]	390 ± 130.4[b]
Amplitude	660-1560	660-1500	180-1140	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. [ab]Mean ± SEM with distinct exponents differ by $P < 0.05$.

blastocoel collapse process, that is, for the MP and MS groups ($P < 0.05$). Our data suggest that these processes caused irreversible damage to their structures, making it impossible for these embryos to maintain their intrauterine development after inovulation. Initially, the embryos could be cultured *in vitro* after warming, for 24 hours, as mentioned by (Slade *et al.*, 1985). However, we chose to inovulate the structures to assess the pregnancy rate. Our results coincide with those observed by (Wilsher, Sandra *et al.*, 2020), who used needles of similar diameter, and reported damage to the embryonic capsule and unsatisfactory pregnancy rates. However, (Ferris *et al.*, 2016) reported a pregnancy rate of 46.7% (7/15), using a larger needle (25G). Thus, it is not possible to make inferences about the possible causes of the differences in pregnancy data observed in the cited articles.

The re-expansion rate after warming was 75% (3/4) for embryos in the MP group and 66.7% (4/6) for those in the MS group. In the NM group, in which there was no previous manipulation, the re-expansion rate was 75% (6/8). These data demonstrate that, in general, the cryopreservation process was efficient in maintaining the osmotic control of blastomeres.

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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