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Vitrifying expanded equine embryos collapsed by blastocoel aspiration is less damaging than slow-freezing

M. Umair [a, *], M. Beitsma [a], M. de Ruijter-Villani [a], C. Deelen [a], C. Herrera [b], T.A.E. Stout [a], A. Claes [a]

[a] Department of Clinical Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands
[b] Clinic of Reproductive Medicine, Vetsuisse Faculty, University of Zurich, Lindau, Switzerland

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ABSTRACT

The cryotolerance of equine blastocysts larger than 300 μm can be improved by aspirating blastocoele fluid prior to vitrification; however, it is not known whether blastocoele aspiration also enables successful slow-freezing. The aim of this study was therefore to determine whether slow-freezing of expanded equine embryos following blastocoele collapse was more or less damaging than vitrification. Grade 1 blastocysts recovered on day 7 or 8 after ovulation were measured (>300–550 μm, n = 14 and > 550 μm, n = 19) and blastocoele fluid was aspirated prior to slow-freezing in 10% glycerol (n = 14), or vitrification (n = 13) in 16.5% ethylene glycol/16.5% DMSO/0.5 M sucrose. Immediately after thawing or warming, embryos were cultured for 24 h at 38 °C and then graded and measured to assess re-expansion. Control embryos (n = 6) were cultured for 24 h following aspiration of blastocoel fluid, without cryopreservation or exposure to cryoprotectants. Subsequently, embryos were stained to assess live/dead cell proportion (DAPI/TOPRO-3), cytoskeleton quality (Phalloidin) and capsule integrity (WGA). For 300–550 μm embryos, quality grade and re-expansion were impaired after slow-freezing but not affected by vitrification. Slow-freezing embryos >550 μm induced additional cell damage as indicated by a significant increase in dead cell proportion and disruption of the cytoskeleton; neither of these changes were observed in vitrified embryos. Capsule loss was not a significant consequence of either freezing method. In conclusion, slow-freezing of expanded equine blastocysts collapsed by blastocoel aspiration compromises post-thaw embryo quality more than vitrification.

1. Introduction

Cryopreservation of equine embryos has several potential benefits, including more efficient use of recipient mares, collection of embryos outside the physiological breeding season, international trade and cryo-banking of embryos, for example, from young mares that are in training and yet to prove themselves [1–3]. Despite these advantages, embryo cryopreservation is not routinely performed in equine practice, mainly due to a lack of super-ovulatory agents and the limited ability of embryos larger than 300 μm to survive cryopreservation. The poor cryotolerance of large embryos is attributed to their physical characteristics [4–7]; the lower surface-to-volume ratio affects the diffusion of cryoprotectants in and out of the cells, and therefore, reduces the rate at which the cryoprotectant reaches equilibrium in and outside the cells [8]; more blastocoele fluid (BF) predisposes larger embryos to ice crystal formation during the freezing process which can, in turn, result in damage to organelles and loss of cellular integrity [7,9]. In addition, the blastocyst capsule increases in thickness during embryo development [10] and is thought to impede the diffusion of cryoprotectants into the embryo [11]. However, it is now known that puncture or puncture accompanied by aspiration of blastocoele fluid prior to vitrification markedly improves the cryotolerance of large embryos [12–15]; on the other hand, to our knowledge the impact of slow-freezing of collapsed large embryos on post-thaw embryo quality has not been reported.

Although the ability to generate a viable pregnancy remains the most important embryo quality parameter for equine practitioners, information on other aspects of embryo quality following slow-freezing or vitrification is limited, even though it is of potential

* Corresponding author. Department of Clinical Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 112, 3584CM, Utrecht, the Netherlands.
E-mail address: m.umair@uu.nl (M. Umair).

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value for refining cryopreservation protocols. In this respect, it is difficult to assess embryo quality immediately post-thaw or warming since the embryos are often still collapsed/shrunken. In addition, transfer of slow-frozen or vitrified embryos results either in success or failure to establish a pregnancy, but gives no additional information concerning embryo quality. By contrast, when embryos are evaluated after 24 h of culture, more detailed information can be recorded about cellular viability, organelle function and whether the capsule remains intact. Furthermore, embryo re-expansion after 24 h of culture (post-thawing or warming) has been proposed as an indicator of embryonic viability [16]. The aim of this study was to compare the impact of slow-freezing with that of vitrification on embryo quality grade, re-expansion, and cellular and capsular integrity of flushed equine expanded blastocysts following puncture and collapse.

2. Materials and methods

2.1. Reproductive management of donor mares

The reproductive tract of donor mares (n = 14; age 14 ± 3 years (mean ± S.D.)) was examined on alternate days by transrectal ultrasonography. Once a growing, dominant follicle (>35 mm) was observed in the presence of uterine edema, ovulation was induced using buserelin acetate (0.33 μg/kg Suprefact®, IM: CHEPLAPHARM, Greifswald, Germany). The day after induction of ovulation, mares were examined and inseminated with at least 500 million progressively motile sperm from a single fertile stallion; ovulation was confirmed the following day.

2.2. Embryo recovery and aspiration of blastocoele fluid

Embryos (n = 33) were recovered at either day 7 or 8 after ovulation by uterine lavage with lactated Ringer's solution (ADCCB2165S, Baxter Healthcare SA, Switzerland) supplemented with 0.5% fetal bovine serum (758093, Greiner Bio-One BV, Alphen aan den Rijn, The Netherlands), as described by Stout [16]. Recovered embryos were identified using a stereomicroscope (SZ-ST, Olympus®, Japan) and washed 4 times in H–SOF (HEPES buffered synthetic oviductal fluid [17]; AVANTEA, Cremona, Italy). Embryos were examined in more detail using an Olympus® IX71 microscope and their size (diameter in μm), quality (grade 1, excellent to 4, degenerated or dead) [18] and developmental stage were recorded. Only grade 1 expanded blastocysts >300 μm were included in this study, while embryos <300 μm (n = 8) were used for other experiments. Embryos (1–3 per mare) were randomly divided into two groups depending on their size (>300–550 μm and >550 μm), and were then assigned into three different treatment groups: control (n = 3, in each size group), slow-freezing (n = 5, >300–550 μm and n = 9, >550 μm), vitrification (n = 6, >300–550 μm and n = 7, >550 μm).

Next, all of the embryos including the controls, were punctured and more than 85% of the blastocoele fluid was aspirated, in a modification of the technique described by Herrera [12]. In brief, embryos were placed in H–SOF (control and slow-freezing group) or PBS without Ca and Mg (D8537, Sigma-Aldrich, Zwijndrecht, The Netherlands: Vitrification group) supplemented with 0.4% BSA (wt/vol, A6003, Sigma-Aldrich) and 0.1% PVP360 (Polyvinylpyrrolidone, wt/vol, 9003-39-8, Sigma-Aldrich), under mineral oil (ART-4008–5P, Coopersurgical, Målov Denmark) at room temperature (RT; 19–21 °C). The embryos were punctured and the blastocyst fluid was aspirated using an Olympus® IX71 microscope equipped with a micromanipulation system (Eppendorf TransferMan NK2, Hamburg, Germany), and either an injection needle (5 μm inner diameter; MIC-50-30, Coopersurgical) or a biopsy needle (15 μm inner diameter; MPB-BS-30, Coopersurgical) for embryos between 300 and 550 μm or larger than 550 μm, respectively.

2.3. Treatment groups

2.3.1. Control

After blastocoele collapse, control embryos (n = 6) were cultured individually in micro drops (50 μL) under mineral oil in a Petri dish (Nunc™ IVF Petri Dishes, 150255, Thermo Scientific™) for 24 h in a Dulbecco's Modified Eagle Medium (DMEM)-based culture medium; DMEM (D6421, Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS, v:v, F4135 Sigma-Aldrich) and Gly-Gln monohydrate (0.5814 mg/mL, G5149 Sigma-Aldrich) at 38 °C in an incubator (Flatbed G185, Coopersurgical, Målov Denmark) containing an atmosphere of 6.5% CO_2 and 5% O_2 in air. Prior to use, the embryo culture medium was equilibrated for 24 h in the incubator (6.5% CO_2 and 5% O_2 in air).

2.3.2. Slow-freezing and thawing

Embryos were slow-frozen using the protocol described by Lazzari et al. [19], with minor modifications. In short, the collapsed embryo was incubated in 5% glycerol (v:v in H–SOF, 49767; Sigma-Aldrich) for 5 min followed by 20 min in 10% glycerol (v:v in H–SOF, 49767; Sigma-Aldrich) at RT (19–21 °C) in a four well dish (Nunc™ IVF multi-dish, 179830 Thermo Scientific™). Next, the embryo was loaded (fluid column, air bubble, droplet containing the embryo, air bubble and fluid column) into a 0.25 mL straw (F01, Agtech, USA). After the open end of the 0.25 mL straw had been heat-sealed, it was partially inserted into an outer, labeled 0.5 mL straw (for identification) and kept in aluminum foil to protect it from light until frozen. All procedures described above were carried out within the 20 min of the 10% glycerol incubation. Subsequently, the straw containing the embryo was loaded into the ethanol bath of a programmable freezing machine (2-stage cascade-cooling circulator; JULABO GmbH, Seelbach, Germany) at −6.5 °C for 5 min, and ice nucleation (seeding) was induced by briefly touching the straw with a metal forceps pre-cooled in liquid nitrogen (LN_2). After observing the crystallization visually, the freezing process was continued according to the slow-freezing program (holding for 5 min at −6.5 °C, followed by cooling at −0.5 °C/minute down to −35 °C) after which the straw was plunged into, and stored in, LN_2 until thawing.

Thawing of slow-frozen embryos was performed by removing the straw containing the embryo from the LN_2, holding it in the air for 8 s and then plunging it into a water bath at RT for 20–30 s. Subsequently, the contents of the straw were expelled into a 3 cm Petri dish. The embryo was then washed by passage through a series of H–SOF solutions containing decreasing concentrations of glycerol (8%, 6%, 4%, 2%, v:v in H–SOF) for 5 min each in a four well dish. After washing out the glycerol and brief examination, embryos were cultured for 24 h, as described for control embryos.

2.3.3. Vitrification and warming

The vitrification of embryos was performed as described by Herrera [12], with minor modifications. All steps were performed at RT. In brief, the collapsed embryo was held in 500 μL embryo holding medium (HM; PBS (D8537) supplemented with 20% fetal calf serum (FCS, v:v, F4135, Sigma-Aldrich) and 5 μg/mL gentamicin (15750–037, Gibco™) for 1 min, and then transferred into 500 μL of vitrification solution 1 (VS1; HM containing 7.5% (v:v) ethylene glycol (EG, 324558, Sigma-Aldrich) and 7.5% (v:v) dimethyl sulphoxide (DMSO, D4540, Sigma-Aldrich)) for 3 min. Next, the embryo was incubated in 500 μL of vitrification solution 2 (VS2; HM containing 16.5% (v:v) EG, 16.5% (v:v) DMSO, and 0.5 M sucrose; S0389, Sigma-Aldrich). Finally, the embryo was loaded onto a hemi-straw

and the excess media was removed to leave just a thin film of VS2 around the embryo. The hemi-straw containing the embryo was then plunged into LN_2, 45 s after the embryo was first placed in VS2. The hemi-straw was then part inserted into a labeled 0.5 mL straw under LN_2 and stored in LN_2 until warming and further processing.

The warming protocol for vitrified embryos was adopted from Herrera [12], with minor modifications, and involved sequential passage at 37 °C through three solutions: 1) Warming solution (WS) 1: Holding medium (HM) supplemented with 0.33 M sucrose, 2) WS2: HM supplemented with 0.2 M sucrose, 3) WS3: HM without sucrose. Briefly, after removing the straw from the LN_2, the outer (labeled) straw was removed, and the hemi-straw immediately submerged in WS1 and the embryo incubated for 5 min at 37 °C; 1 min in well 1 (1000 μL, WS1) and 4 min in well 2 (500 μL, WS1) of a four well plate. Next, the embryo was transferred to WS2 (500 μL, well 3) with a minimum volume of WS1 and incubated for 5 min at 37 °C. Finally, the embryo was transferred to WS3 (500 μL, well 4) and incubated for 5 min. After completion of the warming process and brief examination, embryos were cultured for 24 h, as described for the other groups.

2.4. Post-culture embryo evaluation

Immediately after the 24 h culture, embryos were transferred to a micromanipulation dish containing pre-warmed (37 °C) H–SOF under mineral oil. Embryos were graded (1–4, as described earlier; representative embryos are shown in Fig. 1) and measured (diameter in μm) to calculate embryo re-expansion [embryo re-expansion (%) = (embryo diameter after collapsing, slow-freezing/vitrification and 24 h of culture/embryo original diameter) x 100], and the relationship between embryo grade and embryo re-expansion was assessed. If herniation of part of the embryo through the capsule occurred, a mean of two measurements was taken (perpendicular to each other with one going through both the capsule-contained and herniated area) to give an estimate of the expansion. Embryos that expanded to at least their pre-cryopreservation size were considered to have 're-expanded'. Subsequently, each embryo was washed three times (5 min each) in PBS containing 0.1% (wt/vol) PVP (P0930-50G, Sigma-Aldrich), incubated for 30 min at 37 °C in PBS containing DAPI (4′,6-diamidino-2-phenylindoledihydrochloride, 1:500), and washed again three times (5 min each) in PBS-PVP. Next, each embryo was fixed in 4% paraformaldehyde PBS-PVP for 1 h at RT (19–21 °C) and washed three times (5 min each) in PBS containing 0.1% Triton X100 (PBST), and permeabilized by incubation for 30 min at RT in PBST. Embryos were stained for 1 h at RT in PBS-PVP containing: 1:100 Alexa Fluor™ 568 Phalloidin (A12380, Invitrogen) to stain the cytoskeleton; 1:500 TO-PRO™-3 iodide 642 (T3605, Invitrogen) to counter-stain the nuclei and 1:500 wheat germ agglutinin (WGA-FITC; L4895, Sigma- Aldrich) to stain the capsule and assess its integrity. After washing three times (5 min each) in PBS-PVP, embryos were washed once in a 10 μL drop of an antifade agent (VECTASHIELD; Vector Laboratories, Burlingame, CA, USA) and mounted on a glass slide (Super-frost Plus; Menzel, Braunschweig, Germany) within a spacer (0.12 mm eight-well Secure-Seal Spacer, Molecular Probes) containing 8 μL of antifade agent. After covering with a coverslip (22 × 30mm, $1^{1/2}$ thickness, ERIE Scientific, Portsmouth, USA), each embryo was flattened by gently pressing the coverslip, and the edges were sealed with nail polish. Slides were stored at 4 °C until microscopic analysis. The embryos were protected from light during all incubation steps.

Confocal microscopy was performed using a Nikon A1R/STORM system equipped with four lasers (405 nm for DAPI; 488 nm for WGA; 561 nm for Phalloidin; and 640 nm for TO-PRO ™ -3 iodide) to assess dead cell proportion, cytoskeleton quality and capsule integrity. The dichroic filter was a quad line laser filter (405/488/561/640) and the emission filters were 482/32, 515/30, 595/50 and 700/75 for DAPI, FITC, Alexa Fluor 568 ™ and TO-PRO-3 ™, respectively. Z-stacks (2 μm thickness with top and bottom acquisition defined manually) were acquired using a 20X/0.75 NA (numerical aperture) air immersion lens (1000 μm working distance). Pinhole size was 26.82 μm and images (512x512 pixels) were collected in bidirectional mode with a pixel size of 0.97 μm. 3D rendering and image analysis was performed using NIS elements software (NIKON, Japan, vs 5.21.03). Nuclei were preprocessed by median filtering (count 3) and segmentation thresholds were set to 2047 and 2518 intensity for the DAPI and TO-PRO-3 channels, respectively. The proportion of dead cells was estimated by expressing the DAPI positive cell area as a proportion of the TO-PRO-3® (total cells after fixation) positive area (Supplementary Data File 1). Dead cell proportion was classified as follows: no cell death (0% dead cells), mild cell death (1–10% dead cells), moderate cell death (11–20% dead cells), extensive cell death (>20% dead cells) (Fig. 2; maximum intensity projections of representative embryos). Evaluation of the cytoskeleton of embryos was performed as described by Tharasanit et al. [6]; precise and sharp delineation of actin staining around the cell borders was classified as a grade 1 cytoskeleton, (Fig. 3: A and A 1); less distinct outlining of the cells, combined with occasional small clumps of actin within the cytoplasm, was classified as a grade 2 cytoskeleton (Fig. 3: B and B 1); large areas lacking actin staining, with the visible actin largely agglomerated into intracytoplasmic clumps, resulted in classification as a grade 3 cytoskeleton, (Fig. 3: C and C 1). The blastocyst capsule was classified as either intact (no visible abnormality or puncture hole (Fig. 4 A) or damaged (large rent in the capsule and/or significant herniation of the embryo through the hole (Fig. 4 B).

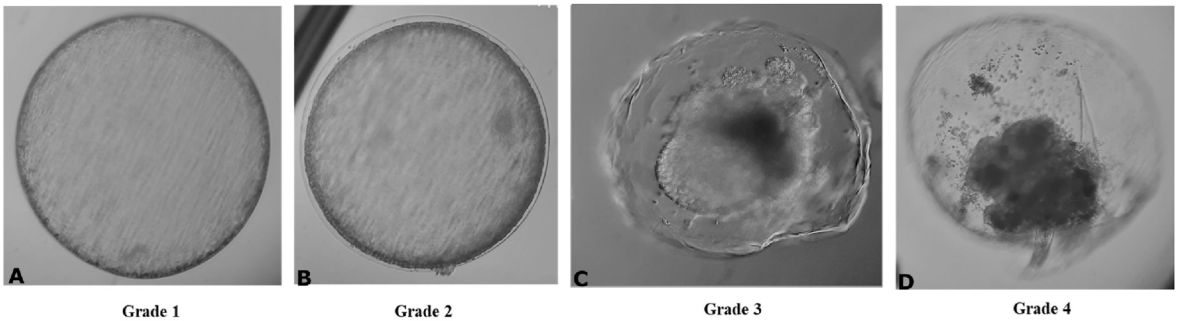

Fig. 1. Embryo grading after thawing/warming and 24 h of culture of expanded equine embryos collapsed by blastocoele puncture prior to cryopreservation. A) Excellent; no visual imperfections, B) Good; minor imperfections, limited separation between trophoblast and capsule, C) Poor; moderate imperfection, D) Dead or degenerated; severe defects, for details [17].

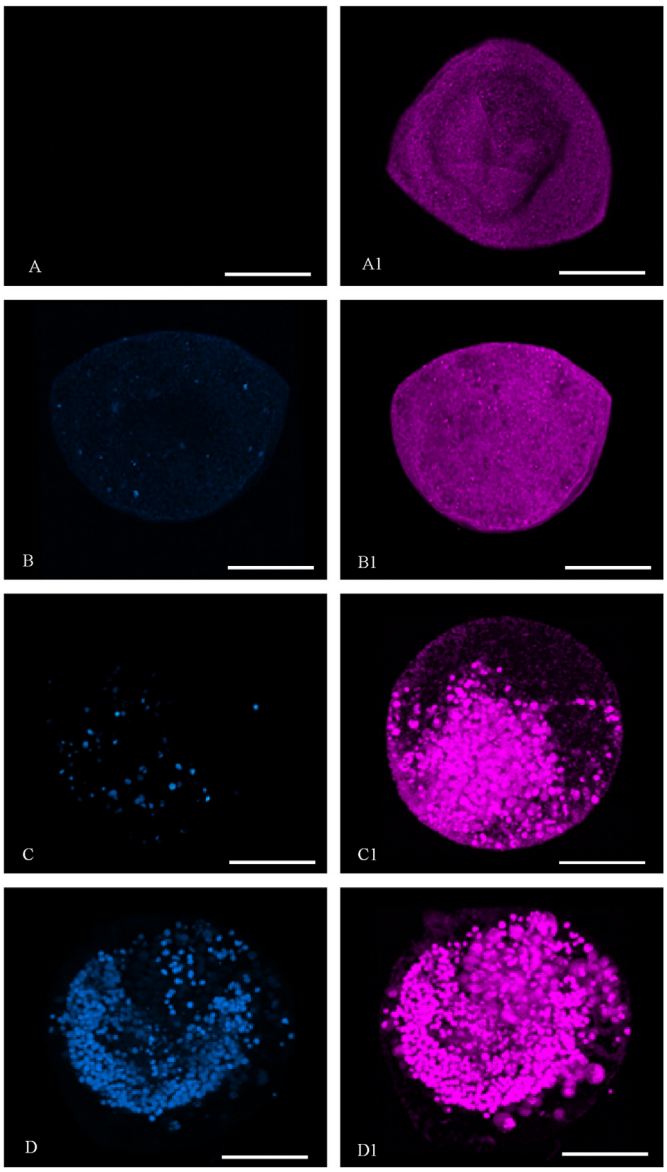

Fig. 2. Dead cell proportion after thawing/warming and 24 h of culture of expanded equine embryos collapsed by blastocoele puncture prior to cryopreservation. Embryos were stained with DAPI before fixation (viability stain; nuclei of membrane damaged cells) and with TO-PRO-3® after fixation (all nuclei). Each image is a maximum intensity projection of an embryo; A) No cell death (0% dead cells); A1) all cells; B) Mild cell death (1–10% dead cells); B1) all cells; C) Moderate cell death (11–20% dead cells); C1) all cells, D) Extensive cell death (>20% dead cells); D1) all cells. Scale bar = 100 μm.

3. Statistical analysis

Differences in embryo re-expansion between control, vitrified and slow frozen embryos were examined using a one-way ANOVA, with a Tukey post hoc test. Kruskal-Wallis tests with Dunn's post hoc testing was used to detect differences in embryo grade, dead cell proportion and cytoskeleton grade between control, slow-frozen and vitrified embryos, while differences in capsule integrity were determined using Fisher's exact test. Student's *t*-test with Welch's correction was used to examine the differences in post-thaw or post-warming embryo expansion between good quality embryos (grade 1 and 2) and poor quality (grade 3 and 4) embryos. Statistical analysis was performed using GraphPad prims software version 8 (GraphPad Software, San Diego, CA, USA). A p-value less than 0.05 was considered to indicate a statistical difference.

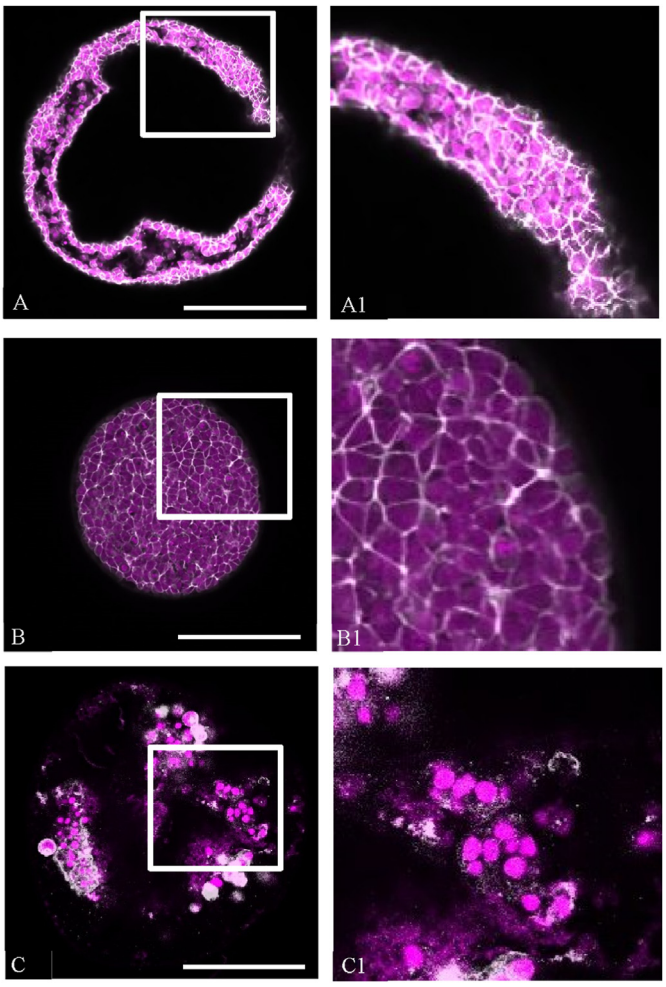

Fig. 3. Cytoskeleton quality after thawing/warming and 24 h culture of expanded equine embryos collapsed by blastocoele puncture prior to cryopreservation. Fixed embryos were stained with Phalloidin-Alexa Fluor® 568 (cytoskeleton stain, grey) and TOPRO-3 (Nuclei, magenta). Each image is from a z-stack of an embryo. A) Grade I cytoskeleton (precise, sharp restriction of actin staining to the cell borders: A1; magnified area represented by white square in A). B) Grade II cytoskeleton (less distinct outlining of the cells, combined with occasional small clumps of actin in the cytoplasm: B1; magnified area represented by white square in B). C) Grade III cytoskeleton (large areas lacking actin staining, with the visible actin largely agglomerated in intracytoplasmic clumps: C1; magnified area represented by white square in C). Scale bar = 100 μm.

4. Results

All frozen and vitrified embryos were still collapsed and shrunken within the capsule immediately after thawing or warming (Supplementary Fig. 1). Categorical data, i.e. the embryo quality grade, dead cell proportion and cytoskeleton quality are shown as proportions (Fig. 5) and median, while continuous data for embryo expansion is shown as mean ± SEM.

4.1. Embryo grade and re-expansion after culture

After 24 h culture, all of the 300–550 μm control embryos were classified as grade 1 (3/3, median = 1). Slow-freezing of embryos led to a significant reduction in embryo quality grade (100% [5/5] grade 3, median = 3) whereas vitrification did not significantly affect embryo quality (67% [4/6] grade 1 and 33% [2/6] grade 3,

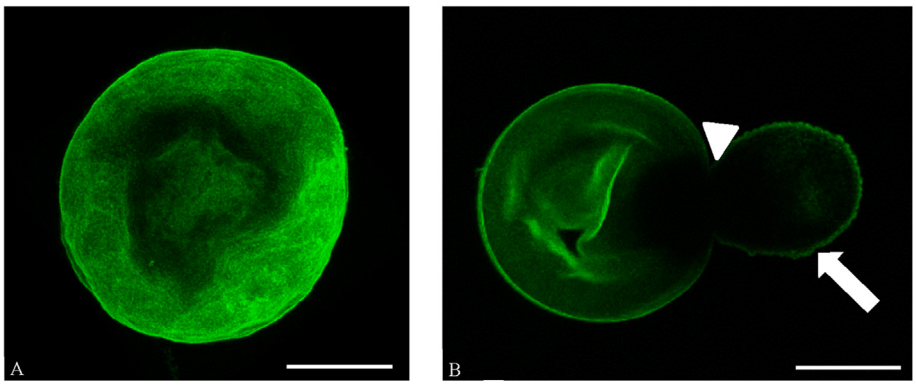

Fig. 4. Integrity of blastocyst capsule after thawing/warming and 24 h culture of expanded equine embryos collapsed by blastocoele puncture prior to cryopreservation. Fixed embryos were stained with WGA-FITC. Each image is a 3D reconstruction of an embryo A) No visible defect in the capsule, B) Large rent in the capsule (arrowhead) and herniation of the embryo from the capsule (arrow); Scale bar = 100 μm.

median = 1: Fig. 5 A). For embryos >550 μm, embryo quality grade was significantly lower after slow-freezing (11% [1/9] grade 2, 78% [7/9] grade 3 and 11% [1/9] grade 4, median = 3: Fig. 6) than vitrification (14% [1/7] grade 1 and 86% [6/7] grade 2, median = 2: Fig. 6). However, neither differed significantly from control embryos (100% [3/3] grade 2, median = 2: Fig. 5 A).

Embryo re-expansion (percentage change in embryo diameter after collapse of the blastocoele, cryopreservation and culture for 24 h) was significantly higher for grade 1 and 2 embryos (137 ± 7%) than grade 3 and 4 embryos (66 ± 9%). Moreover, slow-freezing had a negative impact on embryo re-expansion, which was significantly lower (73 ± 15%) than for vitrified (148 ± 11%) and control (179 ± 4%) embryos between 300 and 550 μm (Fig. 5 B); embryo re-expansion did not differ between control and vitrified embryos. The effect of the freezing method on embryo re-expansion was even more pronounced in larger (>550 μm) embryos; embryo re-expansion was significantly lower in slow-frozen embryos (52 ± 8%) than in control (129 ± 7%) and vitrified embryos (115 ± 3%) but did not differ between the latter 2 groups (Fig. 5 B).

4.2. Dead cell proportion

The freezing method did not influence the proportion of dead cells in embryos between 300 and 550 μm; dead cell proportion was

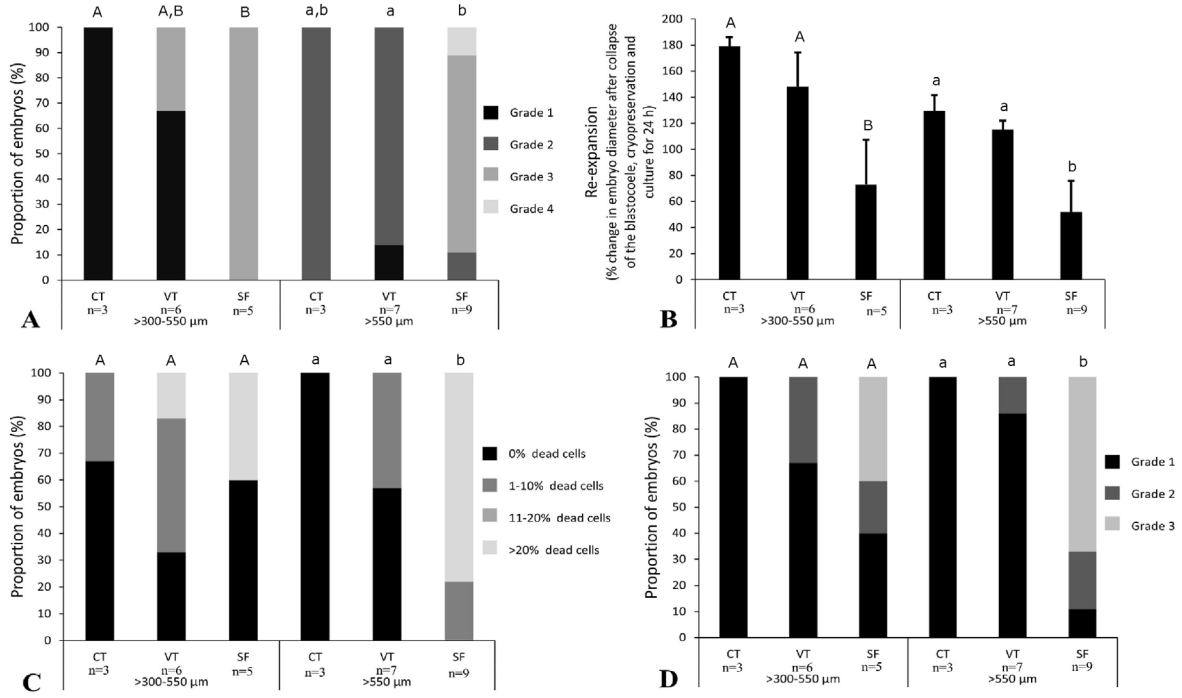

Fig. 5. Embryo quality parameters for control (CT), vitrified (VT) and slow-frozen (SF) expanded equine embryos (>300–550 μm and >550 μm) after thawing/warming and 24 h culture. A) Embryo grade: (graded from 1 (excellent, Black) to 4 (dead, light grey), B) Embryo re-expansion (% change in embryo diameter after collapse of the blastocoele, cryopreservation and culture for 24 h), C) Dead cell proportion: divided into 4 categories; no cell death (0% dead cells), mild cell death (1–10% dead cells), moderate cell death (10–20% dead cells), extensive cell death (>20% dead cells), D) Cytoskeleton quality: graded from 1 (excellent, black) to 3 (poor, light grey). n = number of embryos in each treatment group. Bars in Fig. 5 A, C and D (upper case for embryos >300–550 μm and lower case for embryos >550 μm) with the same superscript do not differ significantly between treatment groups, based on Kruskal-Wallis tests; data are plotted as proportions. Different superscript above the bars in Fig. 5 B (upper case for embryos >300–550 μm and lower case for embryos >550 μm) indicate statistically significant differences in embryo re-expansion; data is depicted as mean ± SEM.

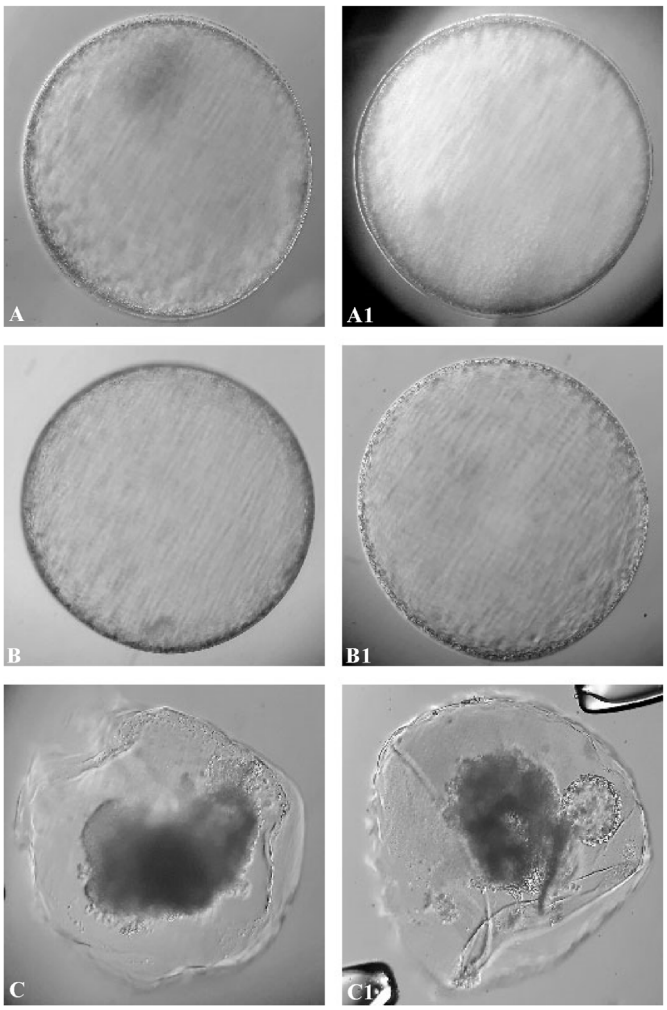

Fig. 6. Appearance of large control, vitrified and slow-frozen equine embryos after 24 h culture subjected to blastocoele collapse prior to cryopreservation. Control embryos (A and A1), Vitrified embryos (B and B2), and slow-frozen embryos (C and C1).

not significantly different between control (67% [2/3] no cell death and 33% [1/3] mild cell death, median = 1), vitrified (33% [2/6] no cell death, 50% [3/6] mild cell death, 0% moderate cell death and 17% [1/6] extensive cell death, median = 2) and slow-frozen (60% [3/5] no cell death and 40% [2/5] extensive cell death, median = 1) embryos (Fig. 5C). By contrast, the freezing method did have a significant impact on cell death in embryos >550 μm, with the proportion of dead cells significantly higher in slow-frozen embryos (0% no cell death, 22% [2/9] mild cell death, 0% moderate cell death and 78% [7/9] extensive cell death, median = 4) than in control (100% [3/3] no cell death, median = 1) and vitrified (57% [4/7] no cell death, 43% [3/7] mild cell death, median = 1) embryos (Fig. 5C).

4.3. Cytoskeleton quality

Although cytoskeleton quality appeared subjectively to be compromised in slow-frozen embryos, there were no statistically verifiable differences in cytoskeleton quality between control (100% [3/3] grade 1, median = 1), slow-frozen (40% [2/5] grade 1, 20% [1/5] grade 2 and 40% [2/5] grade 3, median = 3), and vitrified (67% [4/6] grade 1 and 33% [2/6] grade 2, median = 1) embryos between 300 and 550 μm (Fig. 5 D). Slow-frozen embryos larger than 550 μm did, however, suffer more cytoskeleton disruption (11% [1/9] grade 1, 22% [2/9] grade 2 and 67% [6/9] grade 3, median = 3) than control (100% [3/3] grade 1, median = 1) or vitrified (86% [6/7] grade1 and 14% [1/7] grade 2, median = 1) embryos (Fig. 5 D).

4.4. Embryonic capsule integrity

The integrity of the capsule after thawing or warming was not significantly influenced by the freezing method in either embryo size group (>300–550 μm and >550 μm). Although a large percentage of slow-frozen embryos between 300 and 550 μm had a damaged capsule, there were no significant differences between slow-frozen (20% [1/5] intact and 80% [4/5] damaged capsule), control (100% [3/3] intact capsule) and vitrified embryos (67% [4/6] intact and 33% [2/6] damaged capsule), presumably in part because of the small number of embryos analyzed. Similarly, no significant differences were detected in capsule integrity between control (100% [3/3] intact capsule), slow-frozen (78% [7/9] intact and 22% [2/9] damaged capsule) or vitrified embryos (100% [7/7] intact capsule) larger than 550 μm.

5. Discussion

To the authors' knowledge, this is the first study to examine the impact of slow-freezing on post-thaw quality of expanded horse blastocysts cryopreserved following blastocoele puncture and aspiration. This is surprising since previous studies suggested that, for non-punctured horse embryos, slow-freezing induced less cell damage than vitrification [7]. Surprisingly, in the current study, slow-freezing turned out to be more detrimental than vitrification to embryo grade and re-expansion in embryos >300–550 μm and, in embryos larger than 550 μm also resulted in more extensive cell death and cytoskeleton disruption. Irrespective of embryo size, vitrification was less harmful than slow-freezing in terms of post-warming embryo grade, embryo re-expansion, live-dead cell proportion and cytoskeleton quality, none of which differed significantly to control embryos. We conclude that vitrification appears to be a superior method for cryopreserving *in vivo* developed horse embryos >300 μm in diameter after blastocoele aspiration.

Slow-freezing of collapsed embryos induced more damage than vitrification, while cryo-damage also increased with embryo size as reported previously [6,7]. One of the advantages of vitrification is that it prevents the formation of ice crystals by inducing an instantaneous transition from a liquid to a glass-like state. To achieve this transition, ultra-rapid cooling (20,000 to 30,000 °C/min by plunging embryos directly in LN_2 in an open system; Hemi-straw) with a minimum volume of cryoprotectant solution (<1 μL) is critical. By contrast, the much slower cooling rate (−0.2 to −0.5 °C/min) of the slow-freezing process predisposes embryonic cells to intra-cellular ice crystal formation, particularly if the cryoprotectant has not fully equilibrated between the cells and the medium. Puncturing the embryo and removing blastocoel fluid would be expected to reduce the surface area-to-volume ratio and improve cryoprotectant access, thereby reducing the risk of cryo-damage of large embryos preserved by vitrification. This hypothesis is supported by the poor cryo-tolerance of expanded blastocysts vitrified without blastocoel aspiration [7], and the improved survival when more of the blastocyst fluid is removed from larger blastocysts prior to vitrification [13,14]. Surprisingly blastocoel fluid aspiration did not markedly ameliorate cryo-damage after slow-freezing, despite breaching of the capsule and reduction of embryo volume. Possibly, the small hole made in the capsule and/or the equilibration times used are insufficient to allow adequate influx of cryoprotectant, and efflux of intracellular water.

The other possible reason for the higher levels of damage during

slow freezing, could be a sub-optimal freezing rate during the chosen slow-freezing protocol. Previously, it was reported that the cryo-survival of equine embryos after reducing the volume of blastocoel fluid by dehydration (≈50% reduction in the volume) could be improved by reducing the cooling rate (−0.3 °C/min (−6 °C to −30 °C) and −0.1 °C/min (−30 °C to −33 °C) instead of −0.5 °C/min). The decreased cooling rate increased the total cooling period and allowed larger embryos to dehydrate more completely as the freezing process progressed, and the cryoprotectants would also have more time to reach equilibrium in and outside of the cells [8]. In the current study, the freezing rate was kept at 0.5 °C/min because this works well with small embryos (<250 μm) [4,6,7] and a significant volume (≥85%) of the blastocoel fluid was aspirated before cooling.

It is challenging and difficult to assess embryo quality immediately after thawing or warming, because the embryos are frequently still collapsed/shrunken and separated from the surrounding capsule. However, culturing embryos for 24 h after thawing or warming allows them to re-expand, after which embryo quality parameters, such as embryo grade, re-expansion, cellular viability, cytoskeleton quality and capsule integrity, can be assessed. Moreover, embryo re-expansion during culture is likely to reflect embryo viability and could, therefore, have value in predicting pregnancy outcome after transfer. In our study, embryo re-expansion appeared to be linked to post-thaw/warming embryo quality, with limited re-expansion of poor quality (grade 3 and 4) embryos. Re-expansion of embryos post thawing/warming is positively correlated with the pregnancy outcome for human embryos; the likelihood of obtaining a pregnancy is higher for re-expanded human blastocysts than blastocysts that do not re-expand [20–22].

The likelihood of pregnancy after the transfer of collapsed vitrified embryos has been reported to exceed 70% [13,14]. On the other hand, there is no data on pregnancy rates following transfer of collapsed slow-frozen horse embryos. Although none of the slow-frozen or vitrified embryos were transferred into recipient mares, we presume that the likelihood of obtaining a viable pregnancy after transfer of a slow-frozen, collapsed expanded blastocyst would be low. In particular, with the exception of one large embryo, slow-frozen embryos were predominately classified as grade 3 after 24 h culture. For non-cryopreserved horse embryos, the likelihood of pregnancy is known to be lower after transfer of grade 3 (44%) than grade 1 or 2 (87%) embryos, and recipient mares that receive a grade 3 embryo are more likely to experience subsequent embryonic or fetal loss [23]. Furthermore, slow-frozen embryos larger than 550 μm had a higher percentage of dead cells (>20%). Equine embryos with more than 20% dead cells were previously reported to be non-viable (degenerated) [24]. In conjunction with the high percentage of dead cells, the cytoskeleton of the majority of the slow-frozen large embryos was severely disrupted (grade 3), whereas 86% of the vitrified large embryos had a grade 1 cytoskeleton. Whether these alterations in cytoskeleton integrity in equine embryos influence pregnancy outcome is unknown. However, it has been shown that porcine embryos with a grade 2 cytoskeleton are less likely to result in a pregnancy than embryos with a grade 1 cytoskeleton [25].

6. Conclusion

In conclusion, vitrification should be preferred over slow-freezing as the cryopreservation method of choice for large expanded equine blastocysts subjected to blastocyst puncture and aspiration.

CRediT authorship contribution statement

M. Umair: Conceptualization, Methodology, Data curation, Software, Writing – original draft, preparation, Writing – review & editing. **M. Beitsma:** Methodology, Writing – review & editing. **M. de Ruijter-Villani:** Methodology, Software, Writing – review & editing. **C. Deelen:** Methodology. **C. Herrera:** Methodology, Writing – review & editing. **T.A.E. Stout:** Conceptualization, Supervision, Resources, Writing – review & editing. **A. Claes:** Conceptualization, Software, Supervision, Writing – original draft, preparation, Writing – review & editing.

Declaration of competing interest

Authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2023.02.028.

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