



Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Cryopreservation of Day 8 equine embryos after blastocyst micromanipulation and vitrification

Fabian Diaz^a, Kenneth Bondioli^a, Dale Paccamonti^b, Glen T. Gentry^{c,*}

^a School of Animal Sciences, Louisiana State University Agricultural Center, Baton Rouge, Louisiana, USA

^b Department of Veterinary Clinical Sciences, Louisiana State University School of Veterinary Medicine, Baton Rouge, Louisiana, USA

^c Bob R. Jones–Idlewild Research Station, Louisiana State University Agricultural Center, Clinton, Louisiana, USA

ARTICLE INFO

Article history:

Received 22 June 2015

Received in revised form 28 October 2015

Accepted 28 October 2015

Keywords:

Equine

Cryopreservation

Embryo vitrification

Pregnancy

ABSTRACT

Pregnancy rates after cryopreservation of large equine blastocyst stage embryos have remained lower than other domesticated livestock species. It is generally accepted that the embryonic capsule is the primary barrier to cryoprotectant entry into the embryo proper and techniques need to be developed to circumvent this obstacle. Therefore, the objective of this study was to develop an efficient Day 8 equine embryo cryopreservation protocol through blastocyst micromanipulation and vitrification. Grade 1 and 2 embryos recovered from mares ($n = 15$) 8 days after ovulation were used in these experiments. In experiment 1, the effect of either one- or two-puncture treatments before aspiration of blastocoel fluid and exposure to vitrification solutions was evaluated. No difference was detected in mean embryo volume across treatment groups after exposure to vitrification solutions or after 1, 24, 48, and 72 hours of culture. Percent of embryos re-expanding at 24 hours and percent of embryos showing diameter increase at 48 and 72 hours during *in vitro* culture were 100%, 83%, and 75% compared with 93%, 67%, and 50% for one- and two-puncture treatment groups, respectively. Capsule loss was 25% for one-puncture and 50% for two-puncture treatment groups. In experiment 2, no difference was detected in mean embryo volume for indirect introduction (aspiration of blastocoel fluid + equilibration) and direct introduction (injection of cryoprotectant into blastocoel cavity) treatment groups, after exposure to dilution solution or to culture medium. There was no difference in mean embryo volume for the indirect and direct introduction treatment groups after 1, 24, 48, and 72 hours of culture. Percent of embryos re-expanding at 24 hours and percent of embryos showing diameter increases at 48 and 72 hours during *in vitro* culture were 100%, 76.9%, and 69.2%, respectively, for both treatment groups. Those embryos subjected to the direct introduction treatment had a higher ($P = 0.05$) percent capsule loss (70%) compared with the indirect introduction treatment group (31%). The pregnancy rate after transfer of vitrified expanded Grade 1 blastocysts using the indirect introduction method was 83% (5/6). Three pregnancies were allowed to continue to term and resulted in the birth of three healthy foals. The vitrification protocol used in this study has the potential to become a key tool for the successful cryopreservation of equine expanded blastocysts.

© 2015 Elsevier Inc. All rights reserved.

1. Introduction

The cryopreservation of large equine embryos ($\geq 300 \mu\text{m}$) has typically resulted in pregnancy rates ranging from 0% to 38% [1–6]. The lack of the ability to successfully cryopreserve large equine blastocysts has been attributed to their relatively large blastocoel volume,

* Corresponding author. Tel.: +1 225 683 5848; fax: 225-683-3281.

E-mail address: ggentry@agcenter.lsu.edu (G.T. Gentry).

increased size and cell number, intense mitotic activity, change in expression of membrane protein aquaporin, and development of the acellular glycoprotein capsule [7–13]. Most vitrification studies using large intact equine blastocysts have not resulted in acceptable pregnancy rates. Eldridge-Panuska et al. [3] evaluated the vitrification of expanded blastocysts (mean = 609 μm) using a vitrification protocol that has been used for the successful cryopreservation of equine embryos less than 300 μm , however, no pregnancies were reported in that study. Campos-Chillon et al. [14] used a four-step cryoprotectant addition protocol using ethylene glycol as the cryoprotectant for the vitrification of equine embryos and reported a 35% pregnancy rate in which pregnancies were from embryos that were between 300 and 400 μm in diameter.

The embryonic capsule develops by approximately Day 6.5, soon after the embryo enters the uterus coinciding with the onset of blastulation [15–17]. Although its functions have not been completely elucidated, it is essential for embryonic viability. The capsule appears to play an important role in embryo cell to cell interactions [18], and in addition to the mucin-like glycoproteins, the capsule contains proteins that may be involved in the transport of materials into and out of the developing embryo [19]. Because of the strong and elastic nature of the capsule, it has been proposed that it aids in physical protection of the embryo during the time it is subjected to constant myometrial contractions [20,21], which appear to facilitate maternal recognition during the embryonic mobility phase. This phase occurs between Day 7 and 16 and is required for the embryo to release an antiluteolytic signal, thus preventing CL regression [16]. The embryo mobility response is also thought to be facilitated by the antiadhesive properties of the capsule glycoproteins, which carry a high proportion of sialic acid residues. Loss of sialic acid residues from capsule glycoproteins would terminate the antiadhesion effect and is temporally associated with the fixation of the equine embryo on Day 17, indicating that it is a unique developmentally regulated mechanism of embryo mobility control [18].

The inverse relationship between cryoprotectant permeability and capsule thickness [8] leads us to believe the capsule is the primary barrier to successful cryopreservation of large expanded equine blastocysts [22,23]. Indeed, low permeability has been reported for both the cryoprotectants, glycerol and ethylene glycol, when used on capsulated equine embryos [11,24]. Several different approaches have been applied to overcome the low cryoprotectant permeability of the capsule before cryopreservation but have not resulted in acceptable pregnancy rates. Slow cooling cryopreservation after osmotic-induced dehydration of expanded blastocysts with a reduction of embryo volume by 45% resulted in an overall pregnancy rate of 33% for recipients receiving embryos less than 415 μm transferred in pairs [4]. Legrand et al. [25] reported a 75% pregnancy rate after enzymatic treatment and slow cooling cryopreservation of equine-expanded blastocysts (187–1581 μm), but attempts to replicate these results to date have not been successful [8,26]. Pretreatment of expanded blastocysts (300–1100 μm) with the microfilament inhibitor cytochalasin-B and slow cooling

cryopreservation has resulted in similar pregnancy rates compared with control embryos (42% and 57%, respectively) [26]. The laser-assisted approach to vitrification has been evaluated resulting in a pregnancy rate of 44% at Days 12 to 14, but only one pregnancy remained viable to Day 23 [6]. Therefore, methods to introduce cryoprotectants without negatively impacting subsequent pregnancy rates are needed.

The ability to puncture and manipulate equine oocytes and embryos while still successfully producing offspring has allowed the application of assisted reproductive techniques such as intracytoplasmic sperm injection, embryonic and somatic nuclear transfer, and preimplantation genetic diagnosis [27–29]. The success of these technologies demonstrates that capsule puncture may be a feasible alternative for the cryopreservation of large equine embryos.

In fact, breaching the embryonic capsule followed by extraction of blastocoel fluid has resulted in the successful vitrification of equine-expanded blastocysts with diameters ranging between 300 μm and 650 μm [7]. But to date, limited data exist on larger Day 8 equine embryos. Therefore, the objectives of this experiment were to determine if there were differences in *in vitro* re-expansion and capsule loss of Day 8 equine large expanded blastocysts subjected to vitrification solutions after one or two punctures and either an indirect or direct introduction of cryoprotectants. In addition, *in vivo* survival (by embryo transfer) was tested after indirect introduction of cryoprotectants and vitrification. Therefore, the objective of this study was to develop an efficient Day 8 equine embryo cryopreservation protocol through blastocyst micromanipulation and vitrification.

2. Materials and methods

These experiments were approved by the Louisiana State University Agricultural Center Institutional Animal Care and Use Committee.

2.1. *In vivo* embryo production

Fifteen quarter horse mares were used as embryo donors, and three stallions of known fertility were used as semen donors. The mares ranged in age from 5 to 16 years with body condition score ranging from 5 to 7 (9-point scale), and stallions were 4 and 12 years of age. Stallions were collected on the day of insemination, and all mares were subjected to uterine and ovarian ultrasonography daily using a 5-MHz linear probe (Micromaxx, Sonosite Inc., Bothell, WA, USA). Mares that exhibited a follicle greater than or equal to 34 mm, uterine edema, and no CL were inseminated every other day until ovulation was detected (Day 0).

On Day 8 after ovulation, embryos were recovered nonsurgically as described by Scott et al. [24]. For embryo recovery, a Foley catheter (Agtech Inc., Manhattan, KS, USA) was introduced into the uterine body and the uterus lavaged with 2 L of prewarmed (37 °C) lactated ringer's (Hospira, Lake Forest, IL, USA) supplemented with 1% bovine calf serum (Hy Clone Inc., Logan, UT, USA). Embryos

were collected in a large-volume filter (Miniflush Filter; Minitube, Verona, WI, USA) via gravity flow. Collected embryos were rinsed in 150 to 200 μL drops of holding medium (Syngro holding medium; Bioniche Animal Health, Belleville, Ontario, Canada) four to six times. Morphologic scores from 1 to 4 were assigned to each embryo as described by McKinnon and Squires [30], and embryos were held in holding medium at 37 °C for approximately 15 to 20 minutes until treatments were applied.

2.2. Vitrification solutions

Dulbeccos's phosphate buffered saline (Sigma–Aldrich, St. Louis, MO, USA) without calcium chloride and magnesium chloride was used as the base medium for all vitrification solutions. Dulbeccos's phosphate buffered saline was supplemented with 0.3-mM sodium pyruvate (Sigma–Aldrich), 3.3-mM glucose (Sigma–Aldrich), and 20% fetal bovine serum (vol/vol; Hy Clone Inc.) as described by Eldridge–Panuska et al. [3]. The vitrification protocol used three vitrification solutions, each containing differing concentrations of glycerol and ethylene glycol (Sigma–Aldrich). Vitrification solution 1 (VS1) was composed of 1.4-M glycerol, vitrification solution 2 (VS2) 1.4-M glycerol plus 3.6-M ethylene glycol and vitrification solution 3 (VS3) 3.4-M glycerol plus 4.6-M ethylene glycol. The dilution solution (DS) used for warming embryos contained 0.5-M galactose (Sigma–Aldrich).

2.3. Measurement of embryo diameter

To determine embryo diameter (experiments 1 and 2), digital images were captured on an EVOS microscopy system (Advance Microscopy Group, Mill Creek, WA, USA) during the final 5 seconds of exposure to each solution. Diameter of embryos that maintained a spherical shape was calculated using the mean of two perpendicular measurements, whereas for those with an irregular shape the mean of four perpendicular measurements was used. For experiment 1, embryo measurements were recorded pre-treatment, in each VS, after exposure to DS for 5 minutes, in culture medium for 3 minutes and at 1, 24, 48, and 72 hours of culture. In experiment 2, embryo measurements were recorded after exposure to DS for 5 minutes, in culture medium for 3 minutes, and at 1, 24, 48, and 72 hours of

culture. The mean embryo diameters were transformed to volume using the formula $4/3 \pi r^3$ and expressed as mm^3 .

2.4. Micromanipulation and exposure to vitrification solutions

For both experiments the micromanipulation of embryos was performed using the GeneSearch Embryo Cradle (Genesearch Inc., Bozeman, MT, USA). This pipette is a co-axial microinjection system that consists of a plastic holding pipette and a borosilicate injection pipette introduced from within the lumen of the holding pipette. This configuration allows the aspiration of the fluid from the blastocoel cavity and introduction of cryoprotectant using the same pipette [31]. All micromanipulation was performed on an inverted microscope (Diaphot, Nikon Inc., Melville, NY, USA) at $\times 4$ magnification.

2.4.1. Experiment 1

Collected embryos were transferred into a 500 to 700 μL drop of VS1. Although in VS1, each embryo was held by negative pressure and treatments applied. The one-puncture treatment consisted of the injection pipette being inserted through the embryonic capsule until the trophectoderm was penetrated. For the two-puncture treatment, the injection pipette was inserted completely through the embryo, resulting in two punctures 180° from each other (Fig. 1), then, the injection pipette was retracted into the blastocoel cavity. For both treatments, blastocoel fluid was slowly aspirated until approximately 95% to 99% was removed followed by removal of the injection pipette. The entire manipulation procedure was completed within 3 to 4 minutes (Fig. 2). After administration of treatments, embryos were transferred to VS2 for 5 minutes and VS3 for 45 seconds followed by exposure to DS for 4 minutes and then transferred to culture medium and evaluated at 3 minutes and at 1, 24, 48 and 72 hours of culture. Embryos were exposed to vitrification solutions at room temperature.

2.4.2. Experiment 2

The micromanipulation of collected embryos was performed as described in Section 2.4.1. For the direct introduction treatment, an injection pipette was preloaded with VS1. Once inserted into the blastocoel cavity, the VS1 solution was slowly expelled until embryo diameter reached approximately 110% of pretreatment diameter. Embryos

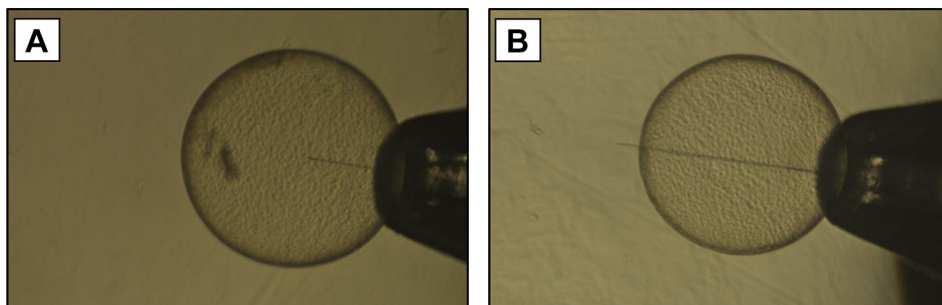


Fig. 1. Panel (A) Single-puncture treatment, Panel (B) Double-puncture treatment.

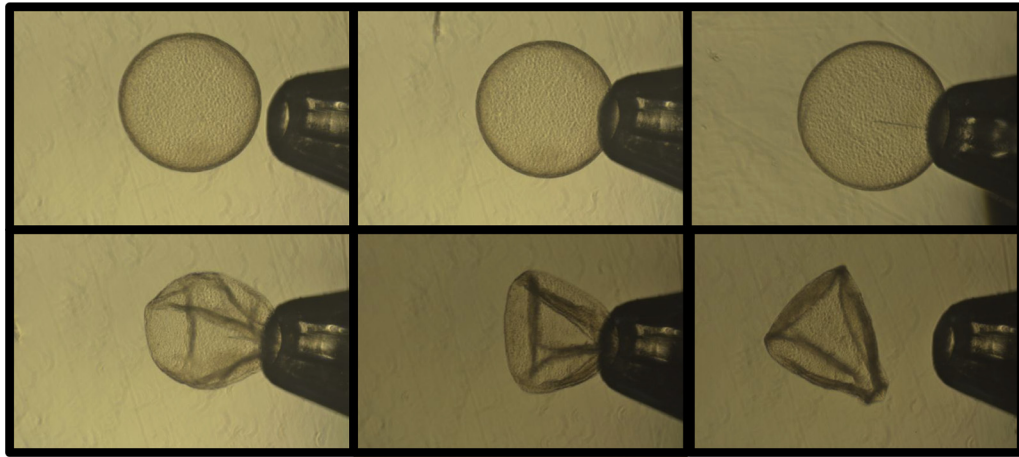


Fig. 2. Capsule puncture and blastocoel fluid extraction sequence.

were allowed to equilibrate for 2 minutes, then the blastocoel fluid was aspirated and the injection pipette removed. For the indirect introduction treatment, the injection pipette was inserted into the blastocoel cavity followed by aspiration of 95% to 99% of the blastocoel fluid. The injection pipette was then removed. For both treatments, all micro-manipulation was performed within 4 minutes, resulting in a total equilibration time in VS1 of 5 minutes.

Embryos were exposed to VS as described in Section 2.4.1. Before plunging in liquid nitrogen (LN), each embryo was loaded onto an open system vitrification device (Cryolock; Biotech Inc., Cumming, GA, USA) in a minimal volume ($<1 \mu\text{L}$) of VS3. Within 45 seconds, the Cryolock containing the embryo was plunged into LN and capped. Vitrified embryos used for embryo transfer were stored in LN tanks for 1 week to 1 month, depending on recipient availability.

For embryo warming, the Cryolock containing each embryo was uncapped under LN and rapidly moved into the prewarmed DS for 4 minutes. The embryo was then transferred into culture medium for culture or to holding medium for subsequent transfer to a recipient mare.

2.5. Embryo in vitro culture

After exposure to vitrification solutions (experiment 1) or vitrification and warming (experiment 2), embryos were cultured *in vitro* for 72 hours as described by Choi et al. (2011; Fig. 3). Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient mixture supplemented with 10% fetal bovine serum and 1% penicillin and/or streptomycin (Life Technologies, Grand Island, NY, USA) was used as the culture medium. For experiment 1, embryos were cultured in 35×100 petri dishes (Becton Dickinson, Franklin Lakes, NJ, USA) containing 3 mL of equilibrated culture medium and covered by 2 mL of mineral oil (Sigma-Aldrich) in an atmosphere of 5% CO_2 , 5% O_2 , and 90% N_2 at 38.2 °C. For experiment 2, embryos were cultured in one well of a 4-well dish (Thermo Scientific, Rochester, NY, USA) in 950- μL culture media covered by 300 μL of mineral oil in an

atmosphere of 5% CO_2 in air at 38.2 °C. For both experiments, embryos were transferred into fresh equilibrated media after 48 hours of culture. Embryo re-expansion and/or collapse and capsule loss were evaluated every 24 hours. Capsule loss was defined as partial or total loss of the embryonic capsule (Fig. 4).

2.6. Transfer of vitrified equine expanded blastocyst

Six Day 8 expanded blastocysts (Grade 1) embryos were vitrified using the indirect introduction technique. The initial diameter of the embryos ranged from 448 μm to 1168 μm . Recipient mares that did not show signs of uterine infection and exhibited normal ovarian activity in the two previous cycles were preselected as recipients.

On the day of transfer, potential recipient mares were evaluated for the presence of an identifiable CL, uterine tone, and the absence of uterine edema. Before transfer, recipient mares received 250 mg of xylazine, iv, (Anased, Lloyd Laboratories, Shenandoah, IA, USA) and 500 mg of flunixin meglumine, iv, (Flunixinamine, Pfizer Inc., New York, NY, USA).

Embryo warming was performed as described in Section 2.4.2. The warmed embryo was removed from DS after 4 minutes and washed four times in holding medium (150 μL) before transfer. Each embryo was loaded into a 0.5-mL sterile plastic straw (Minitube, Ct Verona, WI, USA) between columns of air and medium. A sterile equine embryo transfer pipette covered with a sterile sheath was used for embryo transfer. The embryo was deposited in the uterine body via a transcervical approach.

Pregnancy status was determined 11, 13, 17, 22, and 25 days after ovulation (Fig. 5) through transrectal ultrasonography. Starting on Day 11, altrenogest supplementation was initiated in mares presenting uterine edema of Grade 1 or higher on a scale 0 to 4 (0 being no uterine edema and 4 being maximal uterine edema). Altrenogest supplementation was administered via 22 mg of altrenogest, p.o., (Regu-mate; Intervet, Millsboro, DE, USA) daily until Day 25. Two pregnancies were terminated on Day 25 (Lutalyse; Pharmacia and Upjohn Company, Kalamazoo, MI, USA).

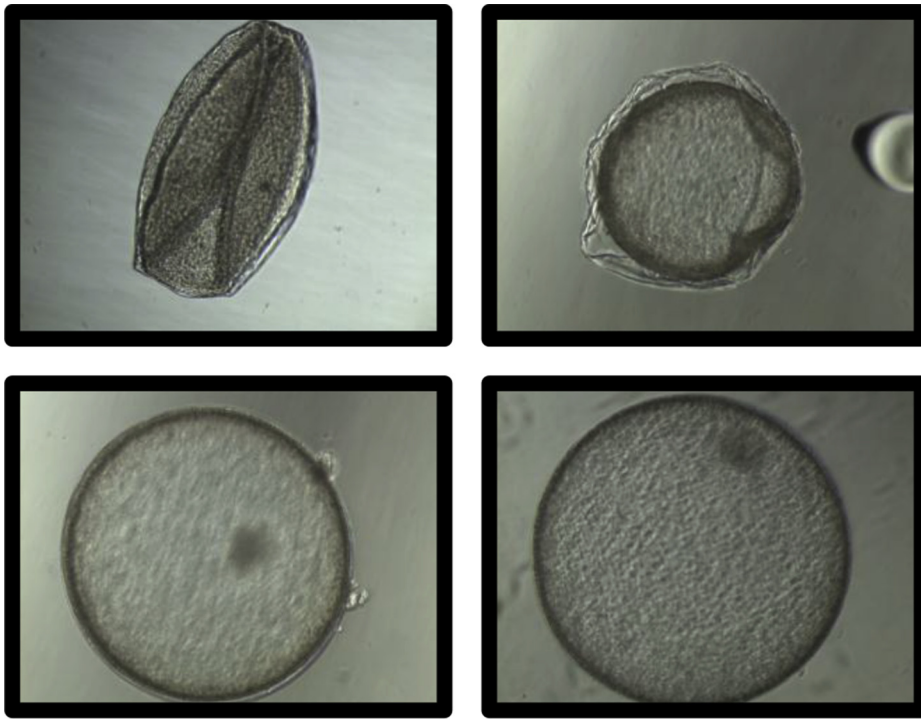


Fig. 3. *In vitro* re-expansion and growth of a vitrified equine-expanded blastocyst.

2.7. Experimental design

2.7.1. Experiment 1

Embryos were subjected to either a one-puncture or two-puncture treatment and exposed to vitrification solutions of a standard equine embryo vitrification protocol. Twenty-four Day 8 equine-expanded blastocysts (Grade 1 and 2) were used in the experiment. Embryos were stratified by diameter across treatments, one-puncture ($n = 12$) or two-puncture ($n = 12$). Embryo volume was determined in each vitrification solution and in culture. Embryo re-expansion and capsule loss was assessed at 24, 48, and 72 hours of *in vitro* culture.

2.7.2. Experiment 2

Twenty-six Day 8 expanded blastocysts (Grades 1 and 2) were subjected to either direct or indirect introduction treatment before vitrification as described in Section 2.4.2.

Embryos were stratified by diameter into direct introduction treatment ($n = 13$) or indirect introduction treatment ($n = 13$). Embryo volume and capsule loss was assessed at 24, 48, and 72 hours of *in vitro* culture. In addition, six Day 8 expanded blastocysts (Grade 1) were vitrified after the indirect introduction treatment and transferred to recipient mares to assess *in vivo* viability.

2.8. Statistical analysis

The embryo volume values were Log transformed for data normalization. A type III test of fixed effects (ANOVA) of the Glimmix procedure was used to determine differences between treatments in embryo volume after exposure to cryoprotectant solutions and during *in vitro* culture. The Log volume of embryos measured over 72 hours of culture was also analyzed using repeated measures ANOVA. Embryo re-expansion rate and capsule loss rate at 24, 48,



Fig. 4. Panel (A) and Panel (B) Partial capsule loss, Panel (C) Total capsule loss.

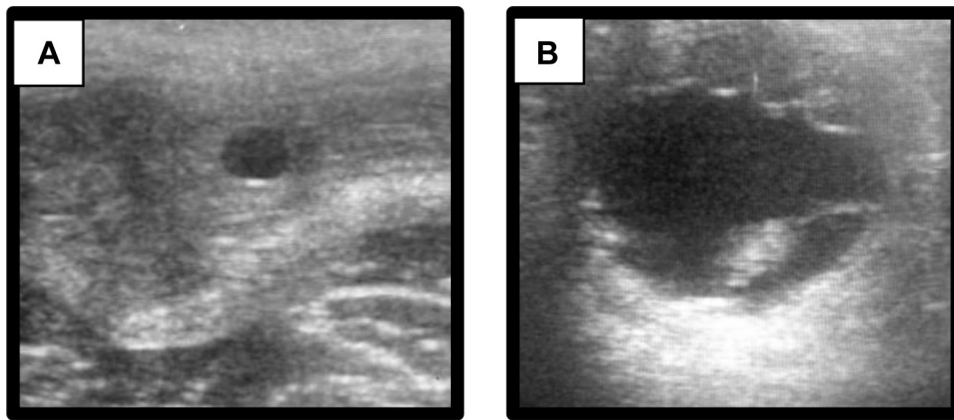


Fig. 5. Panel (A) Day 11 pregnancy, Panel (B) Day 25 pregnancy from a vitrified expanded equine blastocyst.

and 72 hours were analyzed using a chi-square test of independence and logistic regression. Level of significance was set at $P < 0.05$. The Statistical Analysis System (SAS 9.3, SAS Inst. Inc., Cary, NC, USA) software was used for all statistical analyses. Mean embryo volume in this article refers to the geometric least square mean of embryo volume plus the Log least square standard error.

3. Results

3.1. Experiment 1

Embryos, regardless of treatment, exhibited a mean volume reduction of at least 68% after micromanipulation and exposure to VS1. However, no difference was found in mean embryo volume across treatment groups after exposure to any vitrification solutions or DS (Table 1) or at 24, 48, and 72 hours of *in vitro* culture (Table 2). Repeated measures ANOVA of embryo volume over 72 hours of

culture showed no significant interaction of treatments with time ($P = 0.388$). No difference was detected in the percent of embryos re-expanding at 24 hours (100% vs. 93% for one- and two-puncture treatment groups, respectively) or percent of embryos showing increased diameter at 48 and 72 hours during *in vitro* culture (83% and 75% for one-puncture treatment group compared with 67% and 50% for two-puncture treatment group). Likewise, capsule loss was 25% for one-puncture and 50% for two-puncture treatment groups. Differences for re-expansion rate and capsule loss may not have been detected because of the relatively low number of embryos used in the experiment, resulting in a low power of the statistical test. The logistic regression analysis of treatments showed no significant association of embryo volume with embryo re-expansion rate and capsule loss rate ($P = 0.272$ and $P = 0.1115$; $P = 0.579$ and 0.079 for one-puncture and two-puncture treatments, respectively).

3.2. Experiment 2

The embryo volume for indirect and direct introduction treatment groups was not different pretreatment, after exposure to DS for 4 minutes or after exposure to culture

Table 1

Effect of exposure to differing solutions on embryo volume after single- or double-puncture treatments.

Solution ^a	Treatment ^b		P value
	Single puncture ^c	Double puncture ^c	
Pretreatment	0.358 ± 0.14	0.456 ± 0.14	0.60
Vitrification solution 1	0.094 ± 0.16	0.146 ± 0.14	0.41
Vitrification solution 2	0.090 ± 0.15	0.117 ± 0.15	0.60
Vitrification solution 3	0.076 ± 0.16	0.110 ± 0.16	0.47
Dilution solution	0.130 ± 0.14	0.174 ± 0.14	0.53
Culture medium	0.164 ± 0.15	0.216 ± 0.15	0.58

^a Pretreatment, embryo volume before administration of treatments or solutions; vitrification solution 1, 1.4-M glycerol; vitrification solution 2, 1.4-M glycerol + 3.6-M ethylene glycol; vitrification solution 3, 3.4-M glycerol + 4.6-M ethylene glycol; dilution solution, 0.5-M galactose; culture medium, Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient mixture (DMEM/F-12) supplemented with 10% fetal bovine serum and 1% of penicillin and/or streptomycin mixture.

^b Single puncture, injection pipette inserted through the embryonic capsule until the trophectoderm was penetrated with 95% to 99% blastocoel fluid aspirated; double puncture, injection pipette was inserted and passed completely through the embryo, to produce punctures 180° apart with 95% to 99% blastocoel fluid aspirated.

^c Geometric least square (LS) mean of embryo volume (mm³) ± Log LS standard error.

Table 2

In vitro embryo re-expansion and growth after one or two punctures and exposure to vitrification solutions.

<i>In vitro</i> culture (h) ^a	Treatment ^b		P value
	Single puncture ^c	Double puncture ^c	
1	0.136 ± 0.15	0.199 ± 0.15	0.44
24	0.320 ± 0.22	0.232 ± 0.22	0.66
48	0.327 ± 0.26	0.159 ± 0.26	0.41
72	0.361 ± 0.27	0.159 ± 0.29	0.38

^a Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient mixture (DMEM/F-12) supplemented with 10% fetal bovine serum and 1% of penicillin and/or streptomycin was used as the culture medium in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 38 °C.

^b Single puncture, injection pipette inserted through the embryonic capsule until the trophectoderm was penetrated with 95% to 99% blastocoel fluid aspirated; double puncture, injection pipette was inserted and passed completely through the embryo, to produce punctures 180° apart with 95% to 99% blastocoel fluid aspirated.

^c Geometric least square (LS) mean of embryo volume (mm³) ± Log LS standard error.

Table 3

Effect of vitrification after indirect or direct introduction of cryoprotectants on embryo volume.

Solution	Introduction method ^a		P value
	Indirect ^b	Direct ^b	
Pretreatment	0.297 ± 0.15	0.423 ± 0.15	0.48
Dilution solution	0.093 ± 0.16	0.114 ± 0.16	0.70
Culture medium	0.137 ± 0.16	0.178 ± 0.16	0.62

^a Indirect, the injection pipette was inserted into the blastocoel cavity followed by aspiration of 95% to 99% of the blastocoel fluid. The injection pipette was then removed; direct, an injection pipette was preloaded with vitrification solution 1 (VS1). Once inserted into the blastocoel cavity, the VS1 solution was slowly expelled until embryo overexpansion reached approximately 10%. Embryos were allowed to equilibrate for 2 min, then the blastocoel fluid was aspirated and the injection pipette removed.

^b Geometric least square (LS) mean of embryo volume (mm³) ± Log LS standard error.

medium for 3 minutes (Table 3). Mean embryo volume reduction previtrification and postvitrification was 0.297 ± 0.15 versus 0.093 ± 0.16 mm³ (P = 0.06) and 0.423 ± 0.15 versus 0.114 ± 0.16 mm³ (P = 0.004) for indirect and direct introduction treatment groups, respectively. The mean volume for both treatment groups was not different after 1, 24, 48, and 72 hours of *in vitro* culture (Table 4). Repeated measures ANOVA of embryo volume over 72 hours of culture showed no significant interaction of treatments with time (P = 0.784). No difference was detected in embryo re-expansion or diameter increase across treatment groups at 24, 48, and 72 hours of *in vitro* culture. Percent embryo re-expansion was 100% at 24 hours for both treatment groups, and 77% and 69% of embryos for both treatment groups exhibited increased diameter at 48 and 72 hours. However, those embryos subjected to the direct introduction treatment had a higher (P = 0.05) percent capsule loss (70%) compared with the indirect introduction treatment group (31%). Results of logistic regression analysis of treatments showed no significant association of embryo volume with embryo re-expansion rate and capsule loss rate (P = 0.162 and P = 0.573; P = 0.400 and 0.510 for indirect and direct treatments, respectively).

The pregnancy rate after transfer of vitrified expanded blastocysts with a mean diameter of 821 µm

Table 4

Effect of vitrification after indirect or direct introduction of cryoprotectant on *in vitro* embryo re-expansion and growth.

<i>In vitro</i> culture (h)	Introduction method ^a		P value
	Indirect ^b	Direct ^b	
1	0.090 ± 0.14	0.139 ± 0.14	0.36
24	0.619 ± 0.16	0.560 ± 0.16	0.85
48	0.564 ± 0.19	0.655 ± 0.19	0.81
72	0.794 ± 0.223	1.158 ± 0.23	0.62

^a Indirect, the injection pipette was inserted into the blastocoel cavity followed by aspiration of 95% to 99% of the blastocoel fluid. The injection pipette was then removed; direct, an injection pipette was preloaded with vitrification solution 1 (VS1). Once inserted into the blastocoel cavity, the VS1 solution was slowly expelled until embryo overexpansion reached approximately 10%. Embryos were allowed to equilibrate for 2 min, then the blastocoel fluid was aspirated and the injection pipette removed.

^b Geometric least square (LS) mean of embryo volume (mm³) ± Log LS standard error.

Table 5

Pregnancy rates after transfer of vitrified equine-expanded blastocysts.

Mare	Size (µm)	Grade	Altrenogest supplementation ^a	Pregnancy	
				Day 11	Day 25
1	877	1	No	No	No
2	931	1	No	Yes	Yes
3	843	1	Yes	Yes	Yes
4	1168	1	Yes	Yes	Yes
5	659	1	No	Yes	Yes
6	448	1	No	Yes	Yes
Mean	821	1			
Pregnancy rate				83.3%	83.3%

^a Altrenogest supplementation was administered via 22 mg of altrenogest (p.o., Regu-mate, Intervet, Millsboro, DE, USA) daily until Day 25.

(range, 448–1168 µm) using the indirect introduction method was 83% (5/6). Logistic regression analysis showed no significant association of embryo diameter with pregnancy rate (P = 0.782). After embryo transfer, two of the five pregnant mares presented mild signs of uterine edema (Grade 1) at Day 11 after ovulation and were administered altrenogest. On Day 25, the embryonic heartbeat was detected in all five pregnant mares (Table 5). Pregnancies in mares subjected to altrenogest supplementation were terminated, although non-altrenogest supplemented mares completed gestation and foaled resulting in the birth of three healthy foals (Fig. 6).

4. Discussion

Studies have shown embryos greater than 300 µm exposed to 20% ethylene glycol followed by a vitrification solution exhibited volume reductions of 55% within 20 minutes. In contrast, embryos less than 200 µm and 200 to 300 µm reached a volume reduction of 45% and 52%, respectively, within 1 minute [23]. In another study, Young et al. [2] reported the diameter change in embryos with an initial mean diameter of 409 µm and exposed to 4.5-M ethylene glycol at 20 °C to 21 °C for 15 minutes was 60% at 5 minutes, 70% at 10 minutes, and 82% at 15 minutes. Even though both studies were similar in terms of embryo size (>300 µm), cryoprotectant concentrations and



Fig. 6. Healthy foals obtained from vitrified expanded equine blastocysts (Embryo initial diameters from left to right are as follows: 448, 931, 659 µm).

temperature of exposure, they presented differing results in terms of time required for volume reduction.

We believe a rapid embryo volume reduction may be beneficial in allowing an increase in cryoprotectant permeation rate through a reduction in the surface area to volume ratio. In experiment 1 of our study, a reduction of at least 68% of the embryo volume occurred within 5 minutes of embryo puncture, blastocoel fluid extraction, and exposure to VS1. This exposure time to cryoprotectant solutions may have resulted in reduced toxic effects of cryoprotectants as has been reported [32,33]. It has also been shown that the larger the fluid-filled blastocoel cavity, the longer the period required for cryoprotectant concentration equilibration to occur [34]. Because we either breached the capsule or mechanically introduced the cryoprotectant into the blastocoel cavity, cryoprotectant equilibration time should have been reduced. We also showed that embryo volume, as a percentage of the initial embryo volume, was reduced from 26.2% in VS1 to 21.2% in VS3 for the one-puncture treatment and was reduced from 32% in VS1 to 24.2% in VS3 for the two-puncture treatment. These observations suggest that a considerable dehydration beneficial for successful cryopreservation can be achieved when embryos are exposed to a final concentration of 30% to 50% cryoprotectant as described by Leibo and Pool [35].

Because the mean embryo volume for one- and two-puncture treatments was not different during exposure to VS1, VS2, VS3, DS, and culture media in our study, we believe that there is no additional benefit of two-puncture compared with one-puncture treatments. Similarly, a difference was not detected in the mean embryo volume and re-expansion rate for our one- and two-puncture treatment groups during *in vitro* culture. This may be due to the relatively low number of embryos per group ($n = 12$) resulting in low power for statistical comparison. In addition, the two-puncture treatment may result in an increased risk of capsule loss resulting in a reduction of subsequent pregnancy rates. Stout et al. [16] reported that embryos subjected to capsule removal did not result in pregnancies after embryo transfer, showing the importance of the embryonic capsule for successful establishment of pregnancy.

In experiment 2, there was a difference between initial embryo volume and embryo volume after warming of vitrified embryos for direct introduction treatment. Difference was close to significance when analyzing the indirect introduction treatment ($P = 0.06$). From these results, we concluded that embryo volume can be effectively reduced by embryo puncture, blastocoel fluid extraction, and exposure to cryoprotectants. Vanderzwalmen et al. [36] reported that embryo volume reduction may be required to increase embryo viability after vitrification.

It has been suggested that the reduction in survival rates of expanded blastocysts is related to incomplete dehydration and insufficient cryoprotectant permeation into the blastocyst, resulting in ice crystal formation during the vitrification process [37–39]. To overcome this problem in human blastocyst vitrification, the removal of the blastocoel fluid has been tested and has resulted in high survival rates in the range of 87% to 100% [33,36,37,40–42].

In our study, embryo re-expansion was not different across treatment groups during *in vitro* culture at 24, 48, and 72 hours, and both indirect and direct introduction treatments resulted in relatively high re-expansion rates after vitrification and warming, unlike results reported by Hochi et al. [23]. In that study, only 25% of vitrified embryos (mean diameter = 515.4 μm) developed during 48 hours of *in vitro* culture, whereas the remaining 75% presented partial or total degeneration after vitrification. The vitrification protocol used in that study was the same protocol that produced a pregnancy with embryos less than 300 μm [43]. Similarly, Young et al. [2] reported that from eight vitrified embryos (mean diameter = 435 μm) only five were suitable for culture after warming, and after 36 hours of culture, the quality grade of cultured embryos was Grade 2 ($n = 1$), Grade 3 ($n = 1$), and Grade 4 ($n = 3$), with only a 20 μm increase in mean diameter.

In contrast to previous studies, the methods used in our experiments appear to be efficient for the vitrification of expanded equine blastocysts as shown by the resulting high *in vitro* re-expansion rates. Indirect or direct introduction of cryoprotectants resulted in 100% re-expansion rate at 24 hours culture and 69% at 72 hours for both treatments. However, the direct introduction treatment may have negatively impacted chances of pregnancy success by exhibiting higher capsule loss compared with the indirect introduction treatment.

The overexpansion that direct introduction embryos were subjected to at the time of cryoprotectant introduction may have resulted in capsule fracture. Under *in vitro* conditions, embryos were unable to recover, and this resulted in partial or total capsule loss. Data strongly suggest that *in vitro*-produced equine embryos do not develop a normal capsule during culture [44], however, Choi et al. [45] reported that *in vitro* produced-equine blastocysts transferred to recipient mares successfully develop a normal capsule. The latter finding supports the hypothesis that capsule fracture may be reversed *in vivo*. However, further studies are necessary to determine if the capsule damage caused by the micromanipulation procedures performed in these experiments can be reversed *in vivo* and successfully produce pregnancies.

We also have shown that similar re-expansion rates after vitrification can be obtained using either indirect or direct introduction of cryoprotectant. However, we believe that there is no additional benefit of the direct introduction of cryoprotectant because this technique requires higher embryo manipulation skill and results in a significant increase of capsule loss *in vitro*.

In contrast to previous studies, our pregnancy rate (83.3%) was obtained when equine-expanded blastocysts ranging from 448 to 1168 μm (mean = 821 μm) were vitrified after the indirect introduction treatment and transferred into recipient mares. Based on these results, high live foal outcomes might be expected because of the fact that all vesicles continued to develop until the heartbeat stage (Day 25). Logistic regression analysis in experiments 1 and 2 showed no significant association between embryo size and *in vitro* or *in vivo* embryo viability. These results showed that the techniques used in this study help to overcome the long-known inverse relationship between

embryo size and embryo survival when performing equine embryo cryopreservation.

Similar to our study, Choi et al. [7] reported the successful vitrification of equine-expanded blastocysts ranging from 300 to 650 μm by performing embryo puncture and aspirating greater than 70% of the blastocoel fluid before vitrification resulting in a pregnancy rate at Day 5 after transfer of 86% and 71% at Day 25 (heartbeat stage). However, the transfer of a single embryo with a diameter of 780 μm did not result in pregnancy. In the report by Choi et al. [7], five embryos larger than 700 μm were used in the different experiments, and one resulted in vesicle development only (anembryonic vesicle). In our study, we removed approximately 95% to 99% of the blastocoel fluid. Therefore, we hypothesize that complete blastocoel fluid removal results in reduced ice crystal formation within embryonic cells, thereby increasing postwarming embryo viability, which is further supported by the work of others [36,40].

We hypothesize that three important factors may have accounted for the high *in vitro* and *in vivo* embryo viability obtained in our study compared to other studies. The removal of 95% to 99% of blastocoel fluid, the utilization of a three-step cryoprotectant addition protocol consisting of a glycerol and ethylene glycol mixture, and the utilization of an open system vitrification device may have all played key roles. The rapid reduction of blastocoel fluid may decrease the chances of ice crystal formation and reduce the time of exposure to cryoprotectant solutions, which may be beneficial for embryo viability. The three-step cryoprotectant protocol could have positively affected the results of our study, as it has been reported that the use of cryoprotectant mixtures in vitrification procedures is beneficial because of reduced concentrations of cryoprotectants while maintaining the vitrification properties of cryoprotectant solutions thus preventing ice crystal formation [3,46]. In addition, the exposure of embryos to a three-step cryoprotectant addition protocol would allow further embryo extracellular and intracellular dehydration before vitrification compared to a one- or two-step addition protocol. In addition to the known protective capabilities of cryoprotectants, they induce strong dehydration of embryos, therefore increasing the concentration of solutes and facilitating intracellular glass formation during cooling [33,46]. The utilization of an open system vitrification device in which a very low volume of vitrification solution ($<1\ \mu\text{L}$) can be easily obtained might have played an important role in the high viability obtained in our study. The use of an open system vitrification device exhibits multiple advantages including increased cooling and warming rates by using minimal volume of solution [32], preventing the formation of intracellular ice with a smaller amount of intracellular cryoprotectants [32,47], reduction of chilling injury [34,47], shortening the time of exposure to the final cryoprotectant before cooling and after warming [47], and a reduction in extracellular induced injuries [34].

In conclusion, a high pregnancy rate (83.3%) and live healthy foals were the result when large Day 8 equine-expanded blastocysts were vitrified after the indirect cryoprotectant introduction and transferred to recipient mares. We believe the vitrification protocol used in this

study has the potential to become a key tool for the successful cryopreservation of equine-expanded blastocysts.

Acknowledgments

This work was funded by the LSU System/Audubon Center for Research on Endangered Species Collaborative Program. Genesearch Inc. kindly provided the Embryo Cradle and the injection pipettes for embryo micromanipulation in this study.

References

- [1] Slade NP, Takeda T, Squires EL, Eldsen RP, Seidel Jr GE. A new procedure for the cryopreservation of equine embryos. *Theriogenology* 1985;24:45–58.
- [2] Young CA, Squires EL, Seidel GE, Kato H, McCue PM. Cryopreservation procedures for Day 7–8 equine embryos. *Equine Vet J* 1997;29:98–102.
- [3] Eldridge-Panuska WD, Caracciolo di Brienza V, Seidel GE, Squires EL, Carnevale EM. Establishment of pregnancies after serial dilution or direct transfer by vitrified equine embryos. *Theriogenology* 2005;63:1308–19.
- [4] Barfield JP, McCue PM, Squires EL, Seidel GEJ. Effect of dehydration prior to cryopreservation of large equine embryos. *Cryobiology* 2009;59:36–41.
- [5] Bass LD, Denniston DJ, Maclellan LJ, McCue PM, Seidel GE, Squires EL. Methanol as a cryoprotectant for equine embryos. *Theriogenology* 2004;62:1153–9.
- [6] Scherzer J, Davis C, Hurley DJ. Laser-assisted vitrification of large equine embryos. *Reprod Domest Anim* 2011;46:1104–6.
- [7] Choi YH, Velez IC, Riera FL, Roldán JE, Hartman DL, Bliss SB, et al. Successful cryopreservation of expanded equine blastocysts. *Theriogenology* 2011;76:143–52.
- [8] Legrand E, Bencharif D, Barrier-Battut I, Delajarraud H, Corniere P, Fieni F, et al. Comparison of pregnancy rates for days 7–8 equine embryos frozen in glycerol with or without previous enzymatic treatment of their capsule. *Theriogenology* 2002;58:721–3.
- [9] Bruyas JF, Bézard J, Lagneaux D, Palmer E. Quantitative analysis of morphological modifications of day 6.5 horse embryos after cryopreservation: differential effects on inner cell mass and trophoblast cells. *J Reprod Fertil* 1993;99:15–23.
- [10] Stout TAE. Cryopreservation of equine embryos: current state-of-the-art. *Reprod Domest Anim* 2012;47:84–9.
- [11] Gillard Kingma SE, Thibault ME, Betteridge KJ, Schlaf M, Gartley CJ, Chenier TS. Permeability of the equine embryonic capsule to ethylene glycol and glycerol *in vitro*. *Theriogenology* 2011;76:1540–51.
- [12] Betteridge KJ. Equine embryology: an inventory of unanswered questions. *Theriogenology* 2007;68(Suppl 1):S9–21.
- [13] Budik S, Walter I, Tschulenik W, Helmreich M, Deichsel K, Pittner F, et al. Significance of aquaporins and sodium potassium ATPase subunits for expansion of the early equine conceptus. *Reproduction* 2008;135:497–508.
- [14] Campos-Chillon LF, Cox TJ, Seidel Jr GE, Carnevale EM. Vitrification *in vivo* of large equine embryos after vitrification or culture. *Reprod Fertil Dev* 2006;18:151.
- [15] Flood P, Betteridge KJ, Dioece MS. Transmission electron microscopy of horse embryos 3–16 days after ovulation. *J Reprod Fertil Suppl* 1982;32:319–27.
- [16] Stout TAE, Meadows S, Allen WR. Stage-specific formation of the equine blastocyst capsule is instrumental to hatching and to embryonic survival *in vivo*. *Anim Reprod Sci* 2005;87:269–81.
- [17] Betteridge KJ, Eaglesome MD, Mitchell D, Flood PF, Beriault R. Development of horse embryos up to twenty two days after ovulation: observations on fresh specimens. *J Anat* 1982;135:191–209.
- [18] Oriol JG, Sharom FJ, Betteridge KJ. Developmentally regulated changes in the glycoproteins of the equine embryonic capsule. *J Reprod Fertil* 1993;99:653–64.
- [19] Betteridge KJ, Waelchli RO. Equine embryo encapsulation: ephemeral, essential and charismatic. Proceedings of the maternal recognition of pregnancy III Conference, Barbados, West Indies; 2004.
- [20] Leith GS, Ginther OJ. Mobility of the conceptus and uterine contractions in the mare. *Theriogenology* 1985;24:701–11.
- [21] Allen WR. Fetomaternal interactions and influences during equine pregnancy. *Reproduction* 2001;121:513–27.

- [22] Pfaff R, Seidel GE, Squires EL, Jasko DJ. Permeability of equine blastocysts to ethylene glycol and glycerol. *Theriogenology* 1993; 39:284.
- [23] Hochi S, Fujimoto T, Oguri N. Large equine blastocysts are damaged by vitrification procedures. *Reprod Fertil Dev* 1995;7:113–7.
- [24] Scott BR, Carwell DB, Hill RA, Bondioli KR, Godke RA, Gentry GT. Evaluation of capsule permeability in the equine blastocyst. *J Equine Vet Sci* 2012;32:795–8.
- [25] Legrand E, Krawiecki JM, Tainturier D, Corniere P, Delajarraud H, Bruyas JF. Does the embryonic capsule impede the freezing of equine embryos? 5th International symposium on equine embryo transfer, Saari; 2001.
- [26] Maclellan LJ, Carnevale EM, Coutinho da Silva MA, McCue PM, Seidel Jr GE, Squires EL. Cryopreservation of small and large equine embryos pre-treated with cytochalasin-B and/or trypsin. *Theriogenology* 2002;58:717–20.
- [27] Squires EL, Wilson JM, Kato H, Blaszczyk A. A pregnancy after intracytoplasmic sperm injection into equine oocytes matured in vitro. *Theriogenology* 1996;45:306.
- [28] Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, et al. Pregnancy: a cloned horse born to its dam twin. *Nature* 2003;424: 635.
- [29] Choi YH, Gustafson-Seabury A, Velez IC, Hartman DL, Bliss S, Riera FL, et al. Viability of equine embryos after puncture of the capsule and biopsy for preimplantation genetic diagnosis. *Reproduction* 2010;140:893–902.
- [30] McKinnon AO, Squires EL. Morphological assessment of the equine embryo. *J Am Vet Med Assoc* 1988;192:401–6.
- [31] Taylor P, Taylor S, Sansinena M, Godke R. Llama llama pregnancies from vitrified/warmed blastocysts using a novel coaxial cryoprotectant microinjection system. *Reprod Fertil Dev* 2005;18:164.
- [32] Kasai M. Advances in the cryopreservation of mammalian oocytes and embryos: development of ultrarapid vitrification. *Reprod Med Biol* 2002;1:1–9.
- [33] Kader A, Choi A, Orief Y, Agarwal A. Factors affecting the outcome of human blastocyst vitrification. *Reprod Biol Endo* 2009;7:99.
- [34] Kasai M. Simple and efficient methods for vitrification of mammalian embryos. *Anim Reprod Sci* 1996;42:67–75.
- [35] Leibo SP, Pool TB. The principal variables of cryopreservation: solutions, temperatures, and rate changes. *Fertil Steril* 2011;96: 269–76.
- [36] Vanderzwalmen P, Bertin G, Debauche C, Standaert V, van Roosendaal E, Vandervorst M, et al. Births after vitrification at morula and blastocyst stages: effect of artificial reduction of the blastocoelic cavity before vitrification. *Hum Reprod* 2002;17: 744–51.
- [37] Mukaida T, Oka C, Goto T, Takahashi K. Artificial shrinkage of blastocoels using either a micro-needle or a laser pulse prior to the cooling steps of vitrification improves survival rate and pregnancy outcome of vitrified human blastocysts. *Hum Reprod* 2006;21: 3246–52.
- [38] Cho H, Son W, Yoon S, Lee S, Lim J. An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. *Hum Reprod* 2002;17:2419–22.
- [39] Son W, Yoon S, Yoon H, Lee S, Lim J. Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoel. *Hum Reprod* 2003;18: 137–9.
- [40] Hiraoka K, Hiraoka K, Kinutani M, Kinutani K. Blastocoel collapse by micropipetting prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. *Hum Reprod* 2004;19:2884–8.
- [41] Mukaida T, Oka C. Vitrification of oocytes, embryos and blastocysts. *Best Pract Res Clin Obstet Gynaecol* 2012;26:789–803.
- [42] Iwayama H, Hochi S, Yamashita M. In vitro and in vivo viability of human blastocysts collapsed by laser pulse or osmotic shock prior to vitrification. *J Assit Reprod Genet* 2011;28:355–61.
- [43] Hochi S, Fujimoto T, Braun J, Oguri N. Pregnancies following transfer of equine embryos cryopreserved by vitrification. *Theriogenology* 1994;42:483–8.
- [44] Tremoleda JL, Stout TAE, Lagutina I, Lazzari G, Bevers MM, Colenbrander B, et al. Effects of in vitro production on horse embryo morphology, cytoskeletal characteristics, and blastocyst capsule formation. *Biol Reprod* 2003;69:1895–906.
- [45] Choi YH, Harding HD, Hartman DL, Obermiller AD, Kurosaka S, McLaughlin KJ, et al. The uterine environment modulates trophectodermal POU5F1 levels in equine blastocysts. *Reproduction* 2009;138:589–99.
- [46] Ali J, Shelton JN. Successful vitrification of day-6 sheep embryos. *J Reprod Fertil* 1993;99:65–70.
- [47] Vajta G. Vitrification of the oocytes and embryos of domestic animals. *Anim Reprod Sci* 2000;60:357–64.