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Ultra rapid open pulled straw (OPS) vitrification is a perspective for freezing horse embryos

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ABSTRACT

The aim of this research was to evaluate ultra rapid OPS vitrification on the embryo viability. The OPS vitrification technique is comprised of ultra rapid freezing of a small drop in which the embryo is placed. Before the thin straw was plunged into the liquid nitrogen, the embryos were treated with highly concentrated cryoprotectant (CPA) solutions as follows: 18% ethylene glycol (EG), 18% dimethyl-sulfoxide (DMSO) and 0.4 M sucrose. Surgical transfer into the recipient mares and morphologic examination of recollected embryos were used to measure the viability of transferred embryos. The research was performed on Welsh pony mares by collecting the embryos 6.75 days after ovulation. Twenty embryos were vitrified and transferred, four in each recipient mare. At day twelve, nine embryos were recollected after flushing of the recipient uterus (56%, 9/16). In one recipient mare, endometritis was detected when the uterus was flushed. Among the sixteen recollected embryos, seven (44%) had normal morphology and well developed embryonic vesicles. The vitrification procedure used proved to be encouraging.

Key words: horse, embryo, cryopreservation, open pulled straw, vitrification, transfer

Introduction

To sustain cryopreservation, it is necessary to draw out as many molecules of water as possible from the cells of the embryo. To accomplish the task, the embryos are exposed to hyperosmotic solutions of CPA. This is how to diminish the formation and harmful effect of ice crystals on the cell structures. The CPA also diminishes the temperature of

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crystallization. Nevertheless, it is not advisable to expose the embryos to the hyperosmotic solutions of CPA for a long time because of the exaggerated dehydration and toxic effect (BRUYAS, 1999).

In equine embryos, a new membrane, called embryonic capsule is created, after the embryo has reached the uterus in the developmental stage of early blastocyst, five or six days after ovulation. The capsule, formed between the zona pellucida and trophoblast, presents a dense, acellular envelope (BOSQUET et al., 1987) made of mucin like glycoproteins (CHU et al., 1997) which slows down penetration of CPA molecules into the embryo. Embryos in the earlier stage of development do not have (morulae) or have very thin capsules (early blastocysts) and better support the cryopreservation than larger ones (ORIOL et al., 1993; LEGRAND et al., 2000; BRUYAS et al., 2000).

Numerous and voluminous fat vacuoles in cells of young horse embryos are an additional reason for the inferior support of cryopreservation because of heterogeneous crystallization (BRUYAS et al., 2001).

The survival of cryopreserved embryo depends on the CPA used and the developmental stage of the embryo (DOBRINSKY, 1996). LASCOMBES and PASHEN (2000) reported that embryos <220 μm could survive cryopreservation and initiate pregnancies with acceptable rates. The embryos obtained were frozen slowly, employing 10% glycerol (GLY) as the CPA, added in two steps.

OPS vitrification is an ultra rapid procedure applied to overcome the problems of freezing of sensitive embryos. OPS straws are standard 0.25 mL straws with one extremity pulled and thinned by heating. This increases the superficies / volume rate and hastens the cooling rate of the 2 μL drop in which the embryo is contained.

The embryos are dehydrated by short exposure to concentrated CPA solutions and then directly immerged into the liquid nitrogen. Through the ultra rapid cooling rate obtained (20.000 ºC/min) the CPA pass to the stable, amorphous state, which enables the embryos to bypass ice crystal formation and diminish damage (RALL, 1987; SEIDEL, 1996; VAJTA et al., 1998).

Few studies about (ultra rapid) vitrification of horse embryos are available. Although pregnancies are recorded (HOCHI et al., 1994a), there are still no reports about foaling after the embryos treated by this method were transferred (see Discussion).

In this article, the investigation about the efficacy of OPS cryopreservation of horse embryos will be presented.

Materials and methods

Welsh pony mares, 3 to 15 y.o., 250 to 400 kg weight, were used as embryo donors and recipients. To establish the sexual cycle stage, follicular activity and presence of

corpus luteum (CL), gynaecological rectal and ultrasound (5 MHz linear probe, Aloka, Japan) examinations of mares were regularly performed. The same methods were used to examine the uterus and cervix.

During oestrus, the examinations were performed every 24 hours. When the presence of a follicle with a diameter of ≥33 mm was detected, ovulation was induced by an intravenous injection of 15 mg CEG (crude equine gonadotrophin, containing LH). The ovulation was expected 36 h later. Artificial insemination (A.I.) was performed 28 h after hormone application. The single semen dose contained 400 millions spermatozoa diluted in HGLL (Hank's salt, glucose, lactose) + BSA (bovine serum albumin) in a total volume of 10 mL/dose/mare (MAGISTRINI et al., 1992). The semen was obtained from stallions of known fertility, and applied transcervically.

If ovulation was confirmed the day after A.I., the attempt to recover the embryos was planned 6.25 days after. Therefore, the embryos were 6.75 days old when the uterus of the donor mare was flushed. Prostaglandin F2α (0.5 mL, Estrumate, Schering-Plough Vétérinaire, France) was injected immediately after embryo collection.

A nonsurgical transvaginal procedure was used to collect the embryos. For each recovery, 3x0.5 L of Ringer Lactate solution (Hartmann´s solution, Lab. Aguetant, Lyon, France) warmed at 37 °C was used. The recovered medium was, after being filtered, poured into a sterile search dish and then examined under a stereomicroscope (90x). The embryo diameter was measured and the morphologic quality of the embryos was evaluated. Upon location, the embryos were rinsed ten times in F1 solution (phosphate puffered saline PBS + 4 g/L BSA, antibiotics and antifungics, IMV, France). The basic solution consisted of TCM (Tissue Culture Medium 199 Hepes, Sigma M7528) supplemented with 20% (v/v) NBCS (newborn calf serum, Sigma N4762) and 0.1 g/L glutamine (L-glutamine, Sigma G3126), warmed at 35-37 °C. The embryos were rinsed twice for 1 min in a basic solution and then placed in two successive baths containing 7.5% EG (Sigma E9129) + 7.5% DMSO (Sigma D5879) for 3 min, and finally in 2 µL drop containing 18% EG, 18% DMSO and 0.4M sucrose (Sigma S1888). The embryos were loaded by capillarity into the narrow end of an OPS straw (Szigta, Denmark) and plunged into liquid nitrogen. It is advisable to accomplish the final solution step, loading of straws and plunging into liquid nitrogen within 40 s (anyhow in less then 1 min).

For warming, the straw was held in the air at room temperature for 5 s, and then, by capillarity, the embryos were pulled out from the straw into the 800 µL drop containing the basic OPS medium with 0.2 M sucrose. After 1 min, embryos were passed into another well with the same medium for 5 min.

Finally, four embryos of similar size and/or developmental stage and ready to be transferred were loaded into a 0.25 mL french straw (IMV, France). The surgical transfer was used to evaluate the viability of embryos. A surgical right flank incision of the recipient

mares were performed as described by SQUIRES and SEIDEL (1995). The recipient mares were chosen from the mares that spontaneously ovulated five days earlier, which was registered by ultrasound. The embryos were transferred into the proximal one-third of the uterine horn exteriorized through the flank.

Five days later, the twelve day old embryos were transvaginally collected from the recipient mare's uterus. The collection was performed (flushing with physiological solution 0.9% NaCl), regardless of the previous positive or negative ultrasound finding. The recovered embryos were classified as vital if normal morphology, no dark cells in inner cell mass, intact capsule and diameter of at least 1000 μm were found.

Results

Embryo recovery efficacy. This study involved 35 flushings, 6.75 days after ovulation. All recoveries were done by uterine flushing during the breeding season, from the end of March until the beginning of May. Of 35 flushes, 25 (71%) were successful and yielded a total of 26 embryos. The percentage of double ovulation was 2.86% (1/35). There were 29% (10/35) unsuccessful recoveries.

The mean diameter of the embryos (μm). The mean diameter of the embryos collected 6.75 days post-ovulation was 213 μm (minimal value 156 μm; maximal value 356 μm).

Table 1. The developmental stage of embryos obtained by uterine flushing of donor mares before vitrification

Embryonic developmental stage	Number (n)
Morula	2
Early blastocyst	14
Blastocyst	10
Expanded blastocyst	0
Hatched blastocyst	0
Total	26

The stage of embryonic development. Early blastocysts were the dominant developmental stage (54%), followed by a smaller proportion of blastocysts (38%) and quite a small number of morulae (8%). Six embryos were eliminated from the study due to different reasons, such as morphologic imperfections before cryopreservation, losing the embryo during procedures or straw fissures.

The efficacy of transfer of OPS vitrified embryos. Uterine flushing of recipient mares resulted in nine embryos obtained out of twenty embryos transferred. However, embryos lost because of endometritis were excluded from the statistical measurement. Therefore,

the efficacy of OPS vitrification followed by surgical transfer was 56%; seven out of nine embryos obtained were classified as vital (44%).

Table 2. Efficacy of OPS vitrification: developmental stage of vitrified transferred and recovered embryos (at day twelve) and their respective diameter (μm).

Embryos		
Transferred (μm)	Recovered (μm)	Viable
1 M, 3 EB (140-170)	3 (2200, 1000, 320)	2
3 EB, 1 B (150-260)	0	0
4 B (200-320)	3 (6200, 5700, 333)	2
3 EB, 1 B (145-225)	3 (7600, 5000, 1100)	3
1 M, 1 EB, 2 B (140-270)	/ (endometritis)	/
Total = 20 (140-320)	9/16 (56%)	7/16 (44%)

(M - morula, EB - early blastocyst, B - blastocyst)

Discussion

Efficacy of embryo recovery. This study comprised 36 embryo recoveries 6.75 days after ovulation had occurred. All recoveries were performed by transcervical uterine flushing during the breeding season, from the end of March until the beginning of May.

SQUIRES and SEIDEL (1995) obtained 60% recovery efficacy, seven days after ovulation when healthy, young mares were mated with fertile stallions. That is inferior to the results obtained in our study, even though our recoveries were performed earlier (day 6.75). The probable reason for the better efficacy obtained in our study was flushing performed during optimal fertility season. The efficacy of recovery in the LAGNEAUX and PALMER (1991) experiment was 60% when performed 6.75d after ovulation and 100% if flushing was accomplished later.

Despite better results during later embryo recoveries, older embryos are less capable of surviving the freezing because of capsule forming which reduces CPA penetration.

On the other hand, although without or with a very thin capsule, embryo recoveries performed earlier result in a lower recovery rate because the embryos have not yet reached the uterus. Such recoveries make studies more expensive. FERREIRA et al. (1997) and BETTERIDGE (1989) assume that embryos reach the uterus by the 6 and 6-6.5 days respectively; whereas SQUIRES and SEIDEL (1995) recovered embryos already five to six days after ovulation. Embryos reach the uterus at the developmental stage of morula or early blastocyst, that is without or with a very thin capsule.

The mean diameter (μm) and stage of embryonic development. The mean diameter of the embryos collected 6.75 days post-ovulation was 213 μm (minimal value 156 μm; maximal value 356 μm).

Many studies have confirmed that collection of embryos 6.5 days after ovulation is the best for cryopreservation of horse embryos (SQUIRES et al., 1989; LAGNEAUX and PALMER, 1991; MEIRA et al., 1993). The majority of embryos obtained 6.5 days after ovulation are early blastocysts (PALMER and CHAVATTE-PALMER, 2001). This is in accordance with our study, despite the fact that our collections were performed a little later.

On the other hand, HOCHI et al. (1994b) obtained 40% (6/15) expanded blastocysts six days after ovulation which confirmed how difficult it is to obtain morulae and early blastocysts. Nevertheless, by performing the collections six days after ovulation, SQUIRES and SEIDEL (1995) obtained mostly morulae and early blastocysts. They performed flushings 18 hours earlier then we did, which explains the different distribution of developmental stages in our study and more blastocysts than morulae obtained. They also found a linear correlation between the day of collection and embryo diameter.

Several researchers found that equine embryos less than 180-200 μm in diameter better survive freezing and thawing (LASCOMBES and PASHEN, 2000; SEIDEL et al., 1989) and could better result in pregnancy after transfer. Therefore, it would be useful if the size and developmental stage of embryos could be controlled.

BRUYAS et al. (2001) found that there was no correlation between the size, age, developmental stage and number of embryo cells. Therefore, day 6.5 embryos could be bigger than day 7 embryos. Moreover, the embryos recovered at the same time after ovulation, are at different developmental stages. That points out how difficult it is to compose a uniform experimental group of embryos.

LAGNEAUX and PALMER (1991) found uniformity of developmental stages of embryos when ovulation has been induced. A smaller size of embryos will be found if collection is performed earlier. BETTERIDGE (1989) found 150 μm embryos, six days after ovulation. The mean size of embryos in our study is bigger, as we performed the collections 18 hours later, which is quite a long period, especially after blastocoel formation.

PALMER and CHAVATTE-PALMER (2001) found that cell divisions become more frequent after blastocoel formation; LAGNEAUX and PALMER (1991) reported that rapid expansion of embryos occurs after the periviteline space has been lost. MOUSSA et al. (2005b) found 5'bromo-2'deoxy-uridine (BrdU) labelling as a tool for evaluating the quality and viability of equine embryos. BrdU is incorporated into newly synthesised DNA strands which allows the determination of the DNA synthesis and live cell proliferation. They observed that the equine embryo cell cycle is shorter compared with other species and also that equine embryos have a higher cell count at an equivalent developmental stage.

The efficacy of transfer of OPS vitrified embryos. In our study the transfer of OPS vitrified embryos into the recipient mare uterus resulted in 56% (9/16) day-12 embryos recovered and in 44% (7/16) of viable embryos.

The capacity of thawed embryos to initiate a pregnancy in the recipient mare could be the most precise way to measure the efficacy of cryopreservation procedure. This method relies exclusively on embryo quality if rigorous selection of recipient mares has been done.

Several groups of authors have concluded that embryo at morula or early blastocyst stage better support standard cryopreservation (slow freezing) than embryos of diameter greater than 300 μm in the stage of expanded blastocyst (SQUIRES et al., 1989; HOCHI et al., 1994b; LEGRAND et al., 2000; SQUIRES et al., 2003). Standard cryopreservation of smaller equine embryos treated with GLY has resulted in pregnancy rates of 50-64% (SQUIRES et al., 1989; HOCHI et al., 1994b), although day six flushings are significantly less successful (YOUNG et al., 1997; BOYLE et al., 1989; HOCHI et al., 1994b). MACLELLAN et al. (2002) obtained 71% (12/17) Day-16 pregnancies after transfer of <300 μm embryos cryopreserved in two steps: 5% GLY for 10 min and 10% GLY for 20 min and thawed in air for 10 s and than immersed in a 37 °C water bath for 30 s. The obtained results are similar to results of fresh transfer. The percentage of pregnancies after transfer of cryopreserved expanded blastocysts is extremely low (MACLELLAN et al., 2003).

The OPS vitrification procedures (Table 3) comprise the use of high concentrated GLY and/or EG solutions. Besides HOCHI et al. (1994a), CARACCIOLO DI BRIENZA et al. (2004), ELDRIDGE-PANUSKA et al. (2005), CARNEVALE et al. (2004) and HUDSON et al. (2006) who based their experiments on transferring the vitrified / warmed embryos (<300 μm) into the recipient mares and achieved pregnancies, most studies were not successful. Other teams (MOUSSA et al. 2005a; OBERSTEIN et al. 2001; YOUNG et al. 1997; HOCHI et al. 1995) followed similar vitrification protocols, but evaluated their performance on morphology scoring after the embryos were warmed (and cultured).

Apart from HOCHI et el. (1994a) who obtained first Day-60 pregnancies even after nonsurgical transfer of five Day-6 vitrified blastocysts into Day-4 recipient mares, all the other teams who obtained pregnancies after transfer of vitrified / warmed embryos used the same cryopreservation technique including the same CPA. The best results were obtained by HUDSON et al. (2006) with 28/40 (70%) day 16 pregnancies after vitrification and transfer of embryos (185 ± 5 μm) at the stage of morulae and early blastocysts.

Some of the teams, besides standard techniques, prepared embryos to be transferred directly into recipient mares after thawing (CARNEVALE et al., 2004; ELDRIDGE-PANUSKA et al., 2005) and for that reason, besides the final vitrification solution containing the embryos at the central part of the straw, put the dilution solution (0.5 M galactose) at the straw ends. Pregnancy rates did not differ significantly between the two procedures.

Table 3. The vitrification/warming protocols used and their respective success

Authors	Vitrification step 1	Vitrification step 2	Vitrification step 3	Vitrification final step	Thawing step 1	Thawing step 2	Result
Hochi et al. (1994a)	20% EG -10-20'	40% EG, 18% Fi, 0.3 M Su - 1-2'		LN vapour 1', LN immersing	20 °C water bath - 20"	0.5 M Su - 10'	2/5 D60 pregnancies (D6 ET)
Caracciolo di Brienza et al. (2004)	1.4 M GLY - 5'	1.4 M GLY + 3.6M EG - 5'	3.4 M GLY + 4.6M EG - 30"	LN vapour 1', LN immersing	Air - 10"; water bath - 10"	0,25 M Ga - 5'; HM <10'	4/12 D16 gravidities (ET<300 μm)
Hudson et al. (2006)	1.4 M GLY - 5'	1.4 M GLY + 3.6M EG - 5'	3.4 M GLY + 4.6M EG - 1'	LN vapour 1', LN immersing	Air - 10"; water bath - 10"	0.5 M Ga	28/40 (70%) D16 gravidities (ET<300 μm)
Carnevale et al. (2004)	1.4 M GLY - 5'	1.4 M GLY + 3.6M EG - 5'	3.4 M GLY + 4.6M EG - <1'	LN vapour 1', LN immersing	Air-10" water bath - 10"	0.25 M Ga - 5'; HM <10' DT: flicked, room T 4-5'	26/48 (54%) D16 gravidities (ET<300 μm)
Eldridge - Panuska et al. (2005)	1.4 M GLY - 5'	1.4 M GLY + 3.6M EG - 5'	3.4 M GLY + 4.6M EG - <1'	LN vapour 1', LN immersing	Air - 10" 20 °C water bath - 10"	0.25 M Ga - 5'; HM <10'; DT: flicked, room T - 4-5'	30/54 (56%) D16 pregnancies (ET<300 μm)
Moussa et al. (2005a)	7.5% DMSO + 7.5% EG - 3'	18% DMSO + 18% EG + 0.4M Su - <1'		LN immersing	Air - 2 s	0.13 M Su - 1' + 5'; 0.075M Su - 5'; HM - 5'	10/20 E viable after DAPI staining (D6.5 - 6.75 ET)

EG - ethylene glycol; fi - ficoll; HM - holding medium; ET - embryos transferred; GLY - glycerol; Su - sucrose; T - temperature; OPS - open pulled straw method; DMSO - dimethyl-sulfoxide; Ga - galactose; DT - direct transfer; LN - liquid nitrogen

Table 3. The vitrification/warming protocols used and their respective success (continued)

Oberstein et al. (2001)	OPS: 7.5% DMSO + 7.5% EG - 3' (cryoloop 2.5')	OPS: 16.5% DMSO + 16.5% EG + 0.5 M Su - 20-30'' Cryoloop: 17.5% DMSO + 17.5% EG + 1M Su - 20-30''		LN immersing	OPS / cryoloop: 0.25 M Su - 1'	OPS / cryoloop: 0.15 M Su - 5', HM - 5'	49.5% live cells after warming and 20 h culture (ET<300 µm)
Young et al. (1997)	4.5 M EG - 15'	11.9 M EG + 26 mM fi + 0.3 M Ga - 1'		LN vapour 1', LN immersing	Air - 12'';, water bath 12''	5' (0.6M; 0.3 M; 0.15M Ga; HM)	1/8 good morph. after warming (ET>300 µm)
Hochi et al. (1995)	20% EG - 20'	40% EG + 18% fi + 0.3 M Su		LN vapour 1', LN immersing	20 °C water bath - 20''	0.5 M Su - 10';, HM	13/16 (81%) viable after warming and 48h culture (ET<300 µm)

EG - ethylene glycol; fi - ficoll; HM - holding medium; ET - embryos transferred; GLY - glycerol; Su - sucrose; T - temperature; OPS - open pulled straw method; DMSO - dimethyl-sulfoxide; Ga - galactose; DT - direct transfer; LN - liquid nitrogen

Other teams evaluated the embryos without transfer. MOUSSA et al. (2005a) found 50% viable embryos (<20% dead cells) after OPS vitrification and warming. HOCHI et al. (1995) found that 81% of embryos <300 μm developed and re-expanded their blastocoele after culture. Finally, OBERSTEIN et al. (2001) found no differences in vitality and morphology of embryos treated by standard cryopreservation, OPS or cryoloop vitrification and concluded that OPS and cryoloop are similarly effective to conventional freezing methods for small equine embryos (morulae and early blastocysts).

With regard to vitrification of large embryos (>300 μm), CARNEVALE et al. (2004) achieved no pregnancies (0/16) after their transfer. HOCHI et al. (1995) attained significantly lower re-expansion of blastocoele of embryos >300 μm (2/8, 25%) compared to small embryos after warming and 48 h culture. YOUNG et al. (1997) found that six out of eight (75%) embryos >300 μm degenerated after warming and culture. Just two embryos out of eight were viable (just one of them with acceptable morphology) after culture, which proved that horse embryos >300 μm are extremely sensitive to freezing damage.

Our experiment basically obtained similar results as described by other teams which is encouraging. However, due to the small number of treated embryos, they should be confirmed with more transfers. Special emphasis should be placed on the selection of embryos according to their size.

OPS vitrification offers the possibility of transfer of cryopreserved horse embryos, even for commercial application. The vitrification of embryos <300 μm could be used as an alternative procedure to slow cooling methods.

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SAŽETAK

Svrha istraživanju bila je ustanoviti učinkovitost ultrabrze vitrifikacije u otvorenoj rastegnutoj slamci na vitalnost konjskih zametaka, prijenosom vitrificiranih pa otopljenih zametaka u sinkronizirane primateljice. Istraživanje je provedeno na stadu Welsh poni kobila. Davateljicama zametaka maternice su transcervikalno bile ispirane 6,75 dana nakon ovulacije, a zametci su nakon vitrifikacije bili pohranjeni u spremnik s tekućim dušikom. Nakon odmrzavanja, zametci su bili prenijeti u sinkronizirane primateljice. Maternice primateljica bile su ispirane petoga dana nakon prijenosa odmrznutih zametaka. Ukupno je bilo preneseno dvadeset zametaka, a ispiranjem maternica primateljica dobiveno je devet zametaka što iznosi 56% s obzirom da je u jedne primateljice ispiranjem ustanovljen endometritis. Od zametaka dobivenih ispiranjem primateljica, sedam (44%) je imalo morfološki normalno razvijene zametne mjehure. Na osnovi provedenih istraživanja zaključeno je da su rezultati ostvareni vitrifikacijom u otvorenoj rastegnutoj slamci ohrabrujući, ali bi ih s obzirom na mali broj prenijetih zametaka trebalo potvrditi na većem uzorku, posebice sa zametcima odabranoga promjera te brojem ždrebadi.

Ključne riječi: konj, zametak, krioprezervacija, vitrifikacija, transfer