Successful cryopreservation of in vitro derived bovine blastocysts in microcapillary pipette tips

Niasari-Naslaji, A.[1*]; Hansen, P. J.[2]; Moore, K.[2] and Thatcher, W. W.[2]

[1]Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran; [2]Department of Animal Sciences, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, Florida, USA

[*]**Correspondence:** A. Niasari-Naslaji, Department of Clinical Sciences, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran. E-mail: niasari@ut.ac.ir

(Received 25 Apr 2006; revised version 21 Aug 2006; accepted 28 Oct 2006)

Summary

The open pulled straw has been used effectively to vitrify preimplantation embryos because of geometric features that allow rapid rates of temperature exchange. One possible inexpensive alternative to the open pulled straw are commercially-available microcapillary pipette tips commonly used for electrophoresis. The main purpose of this study was to compare the survival rates of in vitro produced blastocysts following vitrification in microcapillary pipette tips and open pulled straws. Two experiments were conducted to investigate the effect of type of carrier, age of expanded blastocyst, and addition of β-mercaptoethanol to post-warming culture medium on survival of vitrified in-vitro derived blastocysts. Expanded blastocysts (Day 7, 0900H and 1900H after insemination; insemination = Day 0 at 0900H) were vitrified while loaded randomly in groups of 4-10 into open pulled straws or pipette tips. Following warming, embryos were cultured in groups of up to 20 in 20 µl microdrops of modified KSOM or modified KSOM containing 100 µM β-mercaptoethanol at 38.5°C in 5% CO_2 in air. Survival after warming was assessed as the percentage of vitrified embryos that re-expanded and the percent that hatched after 48 hrs culture. Post-warming survival rates were not affected by type of carrier or age of the expanded blastocyst ($P>0.05$). The proportions of embryos that re-expanded (55.5%) and hatched (25.7%) were higher ($P<0.01$) for those cultured with β-mercaptoethanol than for those cultured without (re-expansion: 42.4%; hatching: 12.6%). In conclusion, the microcapillary pipette tip represents an inexpensive alternative to the open pulled straw for cryopreservation.

Key words: Vitrification, Open pulled straw, β-mercaptoethanol, Blastocyst, Cattle

Introduction

Several carriers and methods were utilized to store embryos. Standard French mini straws were used for holding the embryos during cryopreservation. These carriers limit the maximum cooling and warming rate to about 2,500°C/min (Palasz and Mapletoft, 1996). The efficiency of vitrifying embryos has been markedly improved by increasing the speed of cooling and warming. Several techniques have been established for this purpose including direct dropping of the embryo into the liquid nitrogen (Riha *et al*., 1991; Yang and Leibo, 1999), using electron microscopic grids (Martino *et al*., 1996; Arav and Zeron, 1997; Park, 1999), cryoloop (Hochi *et al*., 2004; Cai *et al*., 2005), cryotop (Hochi *et al*., 2004; Kelly *et al*., 2006), glass micropipette (Cho *et al*., 2002) and OPS (Vajta *et al*., 1997, 1998, 2000). OPS provide high cooling and warming rates about 20,000°C/min (Vajta *et al*., 1998). This, in turn, improves survival of oocytes and embryos to vitrification (Vajta, 2000). However, construction of open pulled straws by hand is tedious and the potential exists for significant variability in geometric proportions between straws. While OPSs are produced commercially, they are several orders of magnitude more expensive than traditional straws. Potentially, useful alternatives to the open pulled straw exist. In particular, microcapillary pipette tips used for loading samples into electrophoresis gels

share with OPS a narrow diameter and yet is inexpensive.

Age of expanded blastocysts and presence of antioxidant in the culture medium are two potential determinants of embryo survival. Previous results indicate that, for in vitro produced embryos, survival of day 7 expanded blastocysts was superior to that for expanded blastocysts at day 8 (Mahmoudzadeh *et al*., 1995; Sommerfeld and Niemann, 1999). It is not known, however, whether embryos becoming expanded blastocysts earlier on day 7 have greater probability for survival than those becoming expanded blastocysts later in the day. The antioxidant β-mercaptoethanol has been shown to protect from oxidative stress during embryonic development (Caamano *et al*., 1998) and provide a beneficial effect on in vitro blastocyst production (Caamano *et al*., 1998). Moreover, addition of β-mercaptoethanol to the post-warming culture medium has been reported to increase the proportion of vitrified embryos that undergo re-expansion and hatching (Nedambale, 2006).

The major objective of this study was to compare survival of bovine expanded blastocysts following vitrification in OPS versus microcapillary pipette tip. In addition, the effects of the age of expanded blastocyst and the presence of antioxidant in the culture medium on embryo survival were also investigated.

Materials and Methods

Materials

The media HEPES-Tyrodes Lactate (TL), IVF-TL, and Sperm-TL were purchased from Cell and Molecular Technologies Inc. (Lavallete, NJ) and used to prepare HEPES-Tyrodes albumin lactate pyruvate (TALP), IVF-TALP, and Sperm-TALP as previously described (Parrish *et al*., 1986). Oocyte collection medium (OCM) was Tissue Culture Medium-199 (TCM-199) with Hanks salts without phenol red (Atlanta Biologicals, Norcross, GA) supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR) containing 2 IU/ml heparin, 100 IU/ml penicillin-G, 0.1 mg/ml streptomycin, and 1 mM glutamine. Oocyte maturation medium (OMM) was TCM-199 (BioWhittaker, Walkersville, MD) with Earle salts supplemented with 10% (v/v) bovine steer serum, 2 µg/ml estradiol 17-ß, 20 µg/ml bovine FSH (Folltropin-V; Vetrepharm Canada, London, ON), 22 µg/ml sodium pyruvate, 50 µg/ml gentamicin sulfate, and 1 mM glutamine. Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Frozen semen from various bulls was donated by Southeastern Semen Services (Wellborn, FL). Potassium simplex optimized medium (KSOM) that contained 1 mg/ml BSA was obtained from Cell and Molecular Technologies (Lavallete, NJ). On the day of use, KSOM was modified for bovine embryos to produce KSOM-BE2 as described elsewhere (Soto *et al*., 2003). Essentially fatty-acid free (EFAF) BSA was from Sigma (St. Louis, MO).

Embryo production

Oocyte maturation and fertilization were performed using procedures described earlier (Jousan and Hansen, 2004). Cumulus-oocyte complexes (COCs) were obtained by slicing 2- to 10-mm follicles on the surface of ovaries (a mixture of beef and dairy cattle) obtained from Central Beef Packing Co. (Center Hill, FL). Those COCs with at least one complete layer of compact cumulus cells were washed two times in OCM and matured in groups of ten in 50 µl drops of oocyte maturation medium overlaid with mineral oil for 22 hrs at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. Groups of 30 COCs were then transferred to four-well plates containing 600 µl IVF-TALP per well and fertilized with 25 µl (~1 × 10^6) Percoll-purified spermatozoa supplemented with 25 µl 0.5 mmol/l penicillamine, 0.25 mmol/l hypotaurine and 25 µmol/l epinephrine in 0.9% (w/v) NaCl. At 8-12 hrs post-insemination, putative zygotes were denuded of cumulus cells by vortexing with 100 µl hyalurinidase (1000 IU/ml in 1 ml Hepes-TALP medium) for 5 min, washed three times in Hepes-TALP and placed in groups of 30 in 50-µl drops of KSOM-BE2. All drops of embryos were overlaid with mineral oil and cultured at 38.5°C in an

atmosphere of 5% (v/v) CO_2 in humidified air. Expanded blastocysts were collected for vitrification on day 7 (0900H and 1900H) after insemination (Day 0; 0900H).

Vitrification and warming procedure

Expanded blastocysts were washed twice in Hepes-TALP medium. The protocol for vitrification was adapted from Mahmoudzadeh *et al*. (1995). Pre-vitrification (3.6 M ethylene glycol; Fisher Scientific, Fair Lawn, New Jersey, USA) and vitrification [7.2 M ethylene glycol, 18% (w/v) Ficoll (Ficoll PM70, Amersham Biosciences, Uppsala, Sweden), 0.3 M sucrose (Fisher)] solutions were prepared using Tissue Culture Medium-199 with Hank's salt (Hyclone, Logan, UT, USA; base medium) supplemented with 0.035% (w/v) sodium bicarbonate and 20% (v/v) fetal bovine serum (Atlanta Biologicals, Lawrenceville, USA). For each group of blastocysts, two drops of vitrification solution (20 µl each) were prepared. Groups of 4-10 embryos were equilibrated in pre-vitrification solution for 3 min. Then, within 30-45 sec, the embryos were moved to the first and then to the second drop of vitrification solution and finally transferred to either an OPS (Minitube of America, Verona, WI, USA) or an electrophoresis pipette tip (200 µl Multiflex tip, 0.4 mm flat, Catalog No. RN005F-LRS, Dot Scientific, Burton, MI, USA) in the total volume of 2 µl. A photograph of the two carriers is shown in Fig. 1. The external diameters of the far narrow end of 10 open pulled straws and 10 pipette tips were measured using a microscope reticle.

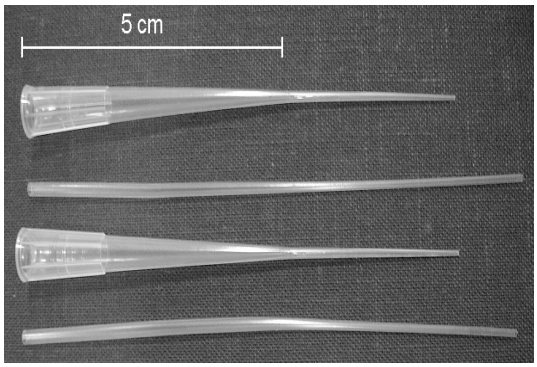

Fig. 1: Carriers used in the experiment. Shown are two examples of the microcapillary pipette (1st and 3rd from top) and an open pulled straw (2nd and 4th from top)

Loading the embryos into OPS was mediated through capillary reaction, while a pipettor was used for loading the embryos into the pipette tips. All procedures were carried out on a plate warmer at 37°C. Following loading, carriers were plunged into liquid nitrogen.

Warming of vitrified embryos was carried at 7 days after vitrification and performed by bending the narrow end of the carrier into a 0.3 M sucrose solution at 37°C and expelling the embryos. Expelling the embryos from OPS was achieved by pressing the thumb at the wide end of the straw. For pipette tips, the liquid was expelled using a pipettor. After 5 min, the embryos were exposed to 0.15 M sucrose for another 5 min, washed three times in Hepes-TALP and placed in culture drops. Both sucrose media were prepared in Tissue Culture Medium-199 with Hank's salt supplemented with 0.035% (w/v) sodium bicarbonate and 20% (v/v) fetal bovine serum.

Experimental design

Experiment 1 used 612 expanded blastocysts to determine the effects of carrier (open pulled straws vs pipette tips) and the age of expanded blastocyst (Day 7 am vs pm) on post-warming survival of vitrified embryos. For experiment 2, 409 expanded blastocysts, in 2×2×2 factorial design, were used to determine effects of carrier, age of expanded blastocyst, and presence of β-mercaptoethanol in the post-warming culture medium (0 or 100 µM; Bio-Rad Laboratories, Richmond CA, USA) on post-warming survival. Expanded blastocysts from each in vitro fertilization run were harvested on day 7 (am and pm), divided into two equal groups, vitrified and stored in open pulled straws or pipette tips. After warming, the embryos were washed three times in Hepes-TALP and transferred into culture medium (KSOM-BE2, Caisson Laboratories Inc, USA) in a maximum group of 20 in 20-µl drops. All drops of embryos were overlaid with mineral oil and cultured at 38.5°C in an atmosphere of 5% (v/v) CO_2 in humidified air. Survival rates were assessed by monitoring re-expansion of the blastocoels and hatching of blastocysts 48

hrs post-warming.

Statistical analysis

The effect of carrier (OPS and pipette tip), age of blastocyst (Day 7 at 0900H and 1900H), ß-mercaptoethanol (with and without) and their possible interactions on the survival rates of blastocysts after warming were analysed by step-wise regression followed by logistic regression using SAS (SAS/STAT, 1996). In this randomized block design experiment, the batch of harvested blastocyst was considered as a block. The external diameter of OPS (n = 10) and microcapillary pipette tip (n = 10) was compared using analysis of variance and F-test.

Results

Across both experiments, the proportion of oocytes that became blastocysts at day 7 after insemination was 21.9 ± 1.5%.

Results on survival after warming for experiment 1 are presented in Table 1. There was no difference in the proportion of embryos that re-expanded or in the proportion of embryos that hatched after warming between blastocysts vitrified in pipette tips as compared to those vitrified with open pulled straws. Similarly, re-expansion and hatching was similar for blastocysts collected on the morning or evening of day 7 and there was no interaction between the main effects of carrier and age.

In experiment 2, there was again no significant effect of type of carrier or age of blastocyst on the percent of embryos that re-expanded or on the percent that hatched (Table 2). Regardless of the type of carrier and age of the blastocyst, addition of β-mercaptoethanol to the post-warming culture medium increased the percent of blastocysts that re-expanded ($P<0.01$) and that hatched ($P<0.01$; Table 2). There were no interactions between β-mercaptoethanol treatment and other main effects.

The external diameter of open pulled straws (n = 10; 502 ± 23.70 μm) was considerably greater than for pipette tips (n = 10; 326 ± 3.05 μm; $P<0.0001$). The variation in external diameter was greater for open pulled straws as compared to pipette tips ($P<0.0001$).

Discussion

The results of the present study indicated that microcapillary pipette tips can be as effective as the open pulled straw for vitrification of bovine embryos. The

Table 1: Post-warming development of in vitro derived, bovine expanded blastocysts, harvested on day 7 (0900H vs 1900H) after insemination (Day 0 at 0900H) and vitrified in either an open pulled straw (OPS) or microcapillary pipette tip

		Expanded blastocysts (n)	Post-warming development (%) after 48 hrs in culture	
			Re-expansion	Hatched
Carrier	OPS	292	38.0	9.3
	Pipette tip	320	44.7	14.7
Time	AM	414	44.0	13.5
	PM	198	36.4	9.1

Table 2: Post-warming development of in vitro derived, bovine expanded blastocysts harvested on day 7 (0900H vs 1900H) after insemination (Day 0 at 0900H), and vitrified in an open pulled straw (OPS) or microcapillary pipette tip and cultured in medium with or without β-mercaptoethanol (β-ME)

		Expanded blastocysts (n)	Post-warming development after 48 hrs in culture (%)	
			Re-expansion	Hatched
β-ME	+	191	55.5[a]	25.7[a]
	-	218	42.4[b]	12.6[b]
Carrier	OPS	201	47.8	17.9
	Pipette tip	208	51.0	21.2
Time	AM	273	53.1	20.9
	PM	136	41.9	16.9

[a, b]values within column with different superscripts differ ($P<0.01$)

advantage of the open pulled straw for vitrification lies in the cooling and warming rates that can be achieved. While standard French ministraws have a maximum cooling and warming rate to about 2,500°C/min (Palasz and Mapletoft, 1996), the open pulled straw exhibits cooling and warming rates of about 20,000°C/min (Vajta *et al*., 1998). It is not surprising that the pipette tips used here were equally effective as the open pulled straw because they too have a very narrow diameter that facilitates heat exchange. As compared to the open pulled straw, the pipette tips offer advantages. The first is in consistency in manufacture. The pipette tips were much more consistent in external diameter than the open pulled straw. The external diameter of the pipette tips was also considerably less than that for open pulled straws; indeed the narrow end of the pipette tip can be fitted into the narrow end of the open pulled straw. This, in turn, might enhance further the rate of cooling and warming rates of pipette tips as compared to open pulled straws. Also, there is a considerable price advantage for the brand of electrophoresis pipette tips used (currently $0.09 US) as compared to the cost of commercially-available open pulled straws (currently $2.90 US). Open pulled straws can be manufactured by hand with modification of French ministraws (currently $0.06 US) but the labor involved with vitrification is thereby increased.

Stage of embryonic development affects the post-warming survival of vitrified bovine embryos. For example, morulae produced in vitro were more sensitive to vitrification than were blastocysts or expanded blastocycts (Mahmoudzadeh *et al*., 1995) and post-warming survival rates of day 7 expanded blastocysts were higher than for non-expanded day 7 blastocysts (Mahmoudzadeh *et al*., 1995). In the present study, however, there was no significant difference in survival between expanded blastocysts collected in the morning or evening of day 7. Thus, differences in competence for survival are slight within this time frame and one could expect equal survivability for blastocysts collected on day 7 regardless of the time of collection.

In the present study, supplementation of culture medium after warming with β-mercaptoethanol increased the proportion of blastocysts that re-expanded and that hatched. This result confirms the recent finding on the beneficial effect of addition of β-mercaptoethanol to post-warming culture medium (Nedambale *et al*., 2006). Following cryopreservation, oocytes and embryos become sensitive to the oxidative stress (Caamano *et al*., 1998), resulting in lipid peroxidation (Guyader-Joly *et al*., 1999), membrane injury and structural destruction (Halliwell *et al*., 1992; Bilodeau *et al*., 2000; Ali *et al*., 2003). Therefore, inhibition of lipid oxidation improves embryo viability after cryopreservation (Tarin and Trounson, 1993). The low-molecular-weight thiol compounds, such as β-mercaptoethanol, maintain the redox state of cells and protect cells against the harmful effects of oxidative injuries (Chance *et al*., 1979; Meister, 1983). As embryos cannot take up cystine which is the oxidized form of cysteine (Takahashi *et al*., 1995), addition of β-mercaptoethanol in animal cell culture protects cysteine from oxidation and consequently enhance its uptake into the cells (Oshima, 1978; Rizzino and Sato, 1978) and preserve the glutathione concentration of in vitro cultured blastocysts (Takahashi *et al*., 1993).

In conclusion, the microcapillary pipette tips represents an inexpensive alternative to the open pulled straws for cryopreservation. Additional studies to confirm the effectiveness of microcapillary pipette tips under a variety of situations, including those in which blastocyst survival after vitrification in open pulled straws is high, is warranted. In addition, effectiveness of vitrification in pipette tips should be confirmed by transferring embryos to recipient animals.

Acknowledgements

Research was supported in part by Grant No. 2004-34135-14715 from the USDA Tropical/Subtropical Agricultural Research Program. The authors thank Amber Brad and William Rembert, for technical assistance; Marshall, Adam and Alex Chernin and employees of Central Beef Packing Co. (Center Hill, FL, USA) for providing ovaries; and Scott A Randell from

Southeastern Semen Services (Wellborn, FL, USA) for donating semen.

References

Ali, AA; Bilodeau, JF and Sirard, MA (2003). Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. Theriogenology. 59: 939-949.

Arav, A and Zeron, Y (1997). Vitrification of bovine oocytes using modified minimum drop size technique (MDS) is effected by the composition and concentration of the vitrification solution and by the cooling conditions. Theriogenology. 47: 341 (abs).

Bilodeau, JF; Chatterjee, S; Sirard, MA and Gagnon, C (2000). Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol. Reprod. Dev., 55: 282-288.

Caamano, JN; Ryoo, ZY and Youngs, CR (1998). Promotion of development of bovine embryos produced in vitro by addition of cysteine and beta-mercaptoethanol to a chemically defined culture system. J. Dairy Sci., 81: 369-374.

Cai, XY; Chen, GA; Lian, Y; Zheng, XY and Peng HM (2005). Cryoloop vitrification of rabbit oocytes. Hum. Reprod., 20: 1969-1974.

Chance, B; Sies, H and Boveris, A (1979). Hydroperoxide metabolism in mammalian organs. Physiol. Rev., 59: 527-605.

Cho, SK; Cho, SG; Bae, IH; Park, CS and Kong, IK (2002). Improvement in post-thaw viability of in vitro-produced bovine blastocysts vitrified by glass micropipette (GMP). Anim. Reprod. Sci., 73: 151-158.

Guyader-Joly, C; Ponchon, S; Durand, M; Heyman, Y; Renard, JP and Menezo, Y (1999). Effect of lecithin on in vitro and in vivo survival of in vitro produced bovine blastocysts after cryopreservation. Theriogenology. 52: 1193-1202.

Halliwell, B; Gutteridge, JM and Cross, CE (1992). Free radicals, antioxidants, and human disease: where are we now? J. Lab. Clin. Med., 119: 598-620.

Hochi, S; Terao, T; Kamei, M; Kato, M and Hirabayashi, M (2004). Successful vitrification of pronuclear-stage rabbit zygotes by minimum volume cooling procedure. Theriogenology. 61: 267-275.

Jousan, FD and Hansen, PJ (2004). Insulin-like growth factor-I as a survival factor for the bovine preimplantation embryo exposed to heat shock. Biol. Reprod., 71: 1665-1670.

Kelly, J; Kleemann, D; Kuwayama, M and Walker, S (2006). Effect of cysteamine on survival of bovine and ovine oocytes vitrified using the minimum volume cooling (MVC) cryotop method. Reprod. Fertil. Dev., 18: 158 (abs).

Mahmoudzadeh, AR; Van Soom, A; Bols, P; Ysebeart, MT and De Kruif, A (1995). Optimization of a simple vitrification procedure for bovine embryos produced in vitro: effect of developmental stage, two-step addition of cryoprotectant and sucrose dilution on embryonic survival. J. Reprod. Fertil., 103: 33-39.

Martino, A; Pollard, JW and Leibo, SP (1996). Effect of chilling bovine oocytes on their developmental competence. Mol. Reprod. Dev., 45: 503-512.

Meister, A (1983). Selective modification of glutathione metabolism. Science. 220: 472-477.

Nedambale, TL; Du, F; Yang, X and Tian, XC (2006). Higher survival rate of vitrified and thawed in vitro produced bovine blastocysts following culture in defined medium supplemented with β-mercaptoethanol. Anim. Reprod. Sci., 93: 61-75.

Oshima, R (1978). Stimulation of the clonal growth and differentiation of feeder layer dependent mouse embryonal carcinoma cells by β-mercaptoethanol. Differentiation. 11: 149-155.

Palasz, AT and Mapletoft, RJ (1996). Cryopreservation of mammalian embryos and oocytes: recent advances. Biotechnol. Adv., 14: 127-149.

Park, SP; Kim, EY; Kim, DI; Park, NH; Won, YS; Yoon, SH; Chung, KS and Lim, JH (1999). Simple, efficient, successful vitrification of bovine blastocysts using electron microscope grids. Hum. Reprod., 14: 2838-2843.

Parrish, JJ; Susko-Parrish, JL; Critser, ES; Eyestone, WH and First, NL (1986). Bovine in vitro fertilization with frozen-thawed semen. Theriogenology. 25: 591-600.

Riha, J; Landa, V; Kneissl, J; Matus, J; Jindra, J and Kloucek, Z (1991). Vitrification of cattle embryos by direct dropping into liquid nitrogen and embryo survival after nonsurgical transfer. Zivoc. Vir., 36: 113-120.

Rizzino, A and Sato, G (1978). Growth of embryonal carcinoma cells in serum-free medium. Proc. Nat. Acad. Sci. USA., 75: 1844-1848.

SAS/STAT (1996). User's guide (version 6.12). Edn., SAS Institute Inc, Cary, NC.,

Sommerfeld, V and Niemann, H (1999).

Cryopreservation of bovine in vitro produced embryos using ethylene glycol in controlled freezing or vitrification. Cryobiology. 38: 95-105.

Soto, P; Natzke, RP and Hansen, PJ (2003). Identification of possible mediators of embryonic mortality caused by mastitis: actions of lipopolysaccharide, prostaglandin F2α, and the nitric oxide generator, sodium nitroprusside dehydrate, on oocyte maturation and embryonic development in cattle. Am. J. Reprod. Immunol., 50: 263-272.

Takahashi, M; Nagai, T; Hamano, S; Kuwayama, M; Okamura, N and Okano, A (1993). Effect of thiol compounds on in vitro development and intra-cellular glutathione content of bovine embryos. Biol. Reprod., 49: 228-232.

Takahashi, M; Nagai, T; Okamura, N and Okano, A (1995). Effect of co-culture cells on the uptake of cystine into bovine embryos. Theriogenology. 43: 332 (abs).

Tarin, JJ and Trounson, AO (1993). Effects of stimulation or inhibition of lipid peroxidation on freezing-thawing of mouse embryos. Biol. Reprod., 49: 1362-1368.

Vajta, G (2000). Vitrification of the oocytes and embryos of domestic animals. Anim. Reprod. Sci., 60-61: 357-364.

Vajta, G; Booth, PJ; Holm, P; Greve, T and Callesen, H (1997). Successful vitrification of early stage bovine in vitro produced embryos with the open pulled straw (OPS) method. Cryo-Letters. 18: 191-195.

Vajta, G; Holm, P; Kuwayama, M; Booth, PJ; Jacobsen, H and Greve, T (1998). Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. Mol. Reprod. Dev., 51: 53-58.

Yang, BS and Leibo, SP (1999). Viability of in vitro-derived bovine zygotes cryopreserved in microdrops. Theriogenology. 51: 178 (abs).