The effect of vitrification in open pulled straws on pregnancy rates after transfer of *in vivo* produced bovine embryos

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ABSTRACT: The aim of this study was to compare pregnancy rates after transfer of *in vivo* produced embryos cryopreserved using open pulled straw (OPS) vitrification (Group V) or conventional freezing method as a control (Group C). Bovine embryos ($Day_{6.5–7.5}$) collected from superovulated cows were classified according to developmental stages and morphological qualities (Grade 1 and 2) before cryopreservation and they were transferred to synchronized heifers after thawing. Pregnancy rates after transfer of morulae, early blastocysts and expanded blastocysts in Group V compared to Group C (54.5%, 12/22 vs. 56.0%, 14/25; 53.3%, 16/30 vs. 58.1%, 18/31 and 57.7%, 15/26 vs. 48.3%, 14/29) were not different ($P > 0.05$). Likewise, pregnancy rates after transfer of embryos of Grade 1 and 2 in Group V compared to Group C (55.1%, 43/78 vs. 54.1%, 46/85 and 36.4%, 12/33 vs. 32.9%, 23/70, respectively) were not different ($P > 0.05$). The study demonstrated similar viability of embryos which were frozen by vitrification or conventional method irrespective of their quality and developmental stage after transfer into recipients.

Keywords: bovine embryo; superovulation; cryopreservation; freezing; thawing; vitrification; embryo transfer

Cryopreservation of embryos has become a widely used method in commercial embryo transfer (ET) because survival rate of cryopreserved/thawed embryos is nearly comparable with fresh embryos (Shaw et al., 2000). Nevertheless conventional cryopreservation is a slow procedure which exposes the embryo at various phases of freezing to the action of many physical, chemical and biological factors. These factors can cause disruption of the *zona pellucida*, cell membranes or cytoskeleton and metabolic disturbances. Such cell damage leads to loss of self-control of the cell and eventually to its death by apoptosis or necrosis (Baguisi et al., 1999). Formation of intracellular ice crystals is considered the most harmful factor which can occur under specific conditions of freezing and thawing with negative affect on recuperation and survival of embryonic cells (Massip et al., 1989; Schiewe, 1991; Leibo and Loskutoff, 1993; Matsuoka et al., 1995; Vajta, 2003; Vajta and Kuwayama, 2006). Vitrification as an alternative method of cryopreservation uses a much more concentrated cryoprotectant and its high cooling rate (20 000–25 000 or more °C/min) prevents the formation of crystalline ice. Instead, the high viscosity of the cryoprotectant forms a solid glass-like mass. Vitrification considerably simplifies and accelerates the cryopreservation process without requiring expensive equipment (Celestinos and Gatica, 2002; Vajta, 2003). This method is also useful for cryopreservation of *in vitro* matured bovine oocytes (Vajta et al., 1998a; Arav et al., 2000; Hurtt et al., 2000; Papis et al., 2000; Asada et al., 2002), of immature GV (germinal vesicle) oocytes (Abe et al., 2005) and of

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in vitro produced (IVP) embryos, which are highly sensitive to freezing due to their high content of lipids (Vajta et al., 1997a,b,c, 1998a, 1999; Lewis et al., 1999; Dattena et al., 2000; Kong et al., 2000; Lazar et al., 2000; Merton et al., 2001; Mtango et al., 2003, Seidel Jr., 2006). In addition vitrification is suitable for freezing of bovine embyos in the precompaction stage (Vajta, 2003) or advanced stages of embryos as *in vivo* or *in vitro* produced hatched blastocysts (Vajta et al., 1997b). Use of vitrification has been increased by the development of vitrification in open pulled straws (OPS). The benefits of this procedure include reduction in cryoprotectant volume in the narrow part of the straw down to 0.5 µl, low heat insulation characteristics of the straw wall, and more than tenfold acceleration of freezing when the straws are immersed into liquid nitrogen (Vajta et al., 1997a,c, 1998a, 1999; Lewis et al., 1999; Dattena et al., 2000; Lazar et al., 2000; and others). Rapid freezing inhibits the formation of ice crystals and inhibits zona fracturing; in addition toxic and osmotic effects at thawing are minimised by immersion of the capillary containing the embryo into a thawing solution. Vajta (2003) presumes the future role of OPS vitrification to be in special areas where the other methods have failed. These areas include cryopreservation of oocytes, handicapped (*in vitro* produced, biopsied or cloned) embryos, small solid tissue particles or embryonic stem cells.

In our previous study (Lopatarova et al., 2002) we tested developmental ability of OPS vitrified embryos only *in vitro* conditions. In this study we have evaluated the survival rate of these embryos in *in vivo* conditions after their transfer into recipients. Namely we have compared pregnancy rates after transfer of *in vivo* produced embryos cryopreserved using OPS vitrification or the conventional freezing method.

MATERIAL AND METHODS

Animals and treatment

Holstein-Friesian cows (n = 36) were superovulated in the luteal phase (start between days 8–12 of oestrous cycle) with eight doses of 480–560 I.U. (25–30 mg) per animal of FSH (Folicotropin, Spofa, Czech Republic) administered at 12-h intervals. Oestrus was induced by double prostaglandin $F_{2\alpha}$ treatment (cloprostenol, Oestrophan, Spofa, Czech Republic) at the time of the fifth and sixth FSH injections. Three artificial inseminations were done at 48, 60 and 72 h after the first administration of $PGF_{2\alpha}$.

Embryo collection and scoring

Embryos were flushed with PBS (Dulbecco's Phosphate Buffered Saline, Live Technologies, Ltd., U.K.) + 1% FCS (foetal calf serum, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic) on day 7 after the first insemination. Flushing as well as embryo isolation were performed as described by Holy et al. (1990).

The embryos obtained were washed with PBS + 10% FCS and were classified according to their developmental stage and quality. Morphologically intact compacted morulae, early blastocysts and expanded blastocyst of quality grade 1 (morphologically intact, even granulation and cell distribution) and quality grade 2 (small deviations, e.g. few excluded blastomeres) were cryopreserved.

Cryopreservation of embryos

Cryopreservation was done using vitrification in open pulled straws (OPS) described by Vajta et al. (1998a). After removing cotton plugs, 0.25-ml French straws (I.M.V., Orsay, France) were softened over a hot plate and pulled manually until the inner diameter decreased from 1.7 to approximately 0.8 mm and the wall thickness in the central part decreased from approximately 0.15 to 0.07 mm. The straws were air-cooled and cut at the narrowest point with a razor blade. Before vitrification, the embryos were firstly incubated in the holding medium (PBS + 10% FCS) supplemented with 7.5% of ethylene glycol and 7.5% dimethyl sulphoxide for 3 min and then transferred into a 20 µl droplet of the holding medium supplemented with of 16.5% ethylene glycol, 16.5% dimethyl sulphoxide, and 0.5 mol/l sucrose. An approximately 1 to 2 µl droplet containing 1–3 embryos was placed in a Petri dish and straws were loaded by means of the capillary effect to be later submerged into liquid nitrogen. The time between the contact of the embryo with the concentrated cryoprotectant solution and plunging into liquid nitrogen did not exceed 25 seconds.

In the frame of conventional freezing method the control embryos were frozen in ethylene glycol

1.5 mol/l ethylene glycol with 0.1 mol/l sucrose, AB Technology, USA (Voelkel and Hu, 1992) as described by Massip et al. (1987) using the HAAKE F 4Q freezer unit.

Thawing and transfer of embryos

Thawing of vitrified embryos was performed by expelling the embryos located toward the end of the straw into 1.2 ml of holding medium containing 0.25 mol/l sucrose. After 1 min all the embryos were transferred into 1.2 ml of holding medium containing 0.15 mol/l sucrose for 5 min and then into sucrose-free holding medium for another 5 min. The temperature of the media was 37°C in each of the steps. Subsequently, the vitrified and the conventionally frozen/thawed (control) embryos were transferred ipsilaterally within 1h after the thawing procedure into synchronized recipient heifers. Pregnancy diagnoses were performed by rectal palpation between days 35 and 50 after the first insemination.

Statistical analysis

The obtained data were analysed by the χ^2 test (2 × 2 contingency tables). The results of the analyses are summarised in Tables 1 and 2.

RESULTS

Table 1 summarizes pregnancy rates after transfer of embryos frozen/thawed using OPS vitrification (Group V) as well as embryos conventionally frozen/thawed embryos (Group C) at different stages of development. The pregnancy rates after transfer of morulae and early blastocysts in Group V compared to Group C (54.5%, 18/31 vs. 56.0%, 14/25 and 53.3%, 16/30 vs. 58.1%, 18/31) did not differ ($P > 0.05$). Although the conception rate after transfer of expanded blastocysts in Group V was higher than in Group C (57.7%, 15/26 vs. 48.3%, 14/29) the difference was not significant ($P > 0.05$). Pregnancy rates after transfer of embryos of qual-

Table 1. Conception rates after transfer of $D_{6.5–7.5}$ bovine embryos of different quality according to the freezing method

Stage of development	Freezing	Embryos transferred	Pregnancies	
		n	*n*	%
Morula	vitrification	22	12	54.5[a]
	conventional	25	14	56.0[b]
Early blastocyst	vitrification	30	16	53.3[c]
	conventional	31	18	58.1[d]
Expanded blastocyst	vitrification	26	15	57.7 [e]
	conventional	29	14	48.3 [f]

a:b, c:d, e:f = $P > 0.05$

Table 2. Conception rates after transfer of $D_{6.5–7.5}$ bovine embryos of different developmental stage according to the freezing method

Quality grade	Freezing	Embryos transferred	Pregnancies	
		n	*n*	%
1	vitrification	78	43	55.1[a]
	conventional	85	46	54.1[b]
2	vitrification	33	12	36.4[c]
	conventional	70	23	32.9[d]

a:b, c:d = $P > 0.05$

ity grade 1 and 2 in Group compared to Group C are shown in Table 2. The values after transfer of Grade 1 embryos was similar (55.1%, 43/78 vs. 54.1%, 46/85; $P > 0.05$). The values of pregnancy rates were lower after transfer of Grade 2 embryos but the values in Group V compared to Group C (36.4%, 12/33 vs.32.9% 23/70) also were not different ($P > 0.05$).

DISCUSSION

The benefits of vitrification in OPS result from a considerable thinning of the straw wall from 1.7 to 0.8 mm, which markedly reduces the thermal insulation effect, as well as from a considerable reduction of the inner diameter of OPS from 0.85 to 0.07 mm, which minimises the volume of the cryoprotectant, enhances capillary lift of the embryo and helps to maintain a constant level of the cryoprotectant solution in the capillary. Temperature drop from 0°C to –196°C is accelerated by direct contact of the cryoprotectant solution with liquid nitrogen to 16 700–22 500°C per min, which is a tenfold of the freezing speed than in sealed 0.25-ml straws. The efficacy of the OPS vitrification is further enhanced by direct contact of the embryos with the thawing solution. It decreases exposure to its toxic as well as other adverse effects on the embryos. Such rehydration of embryos occurs immediately after immersion into the thawing solution (Vajta et al., 1997a, 1998a). Another benefit of OPS is a significant reduction or complete elimination of *zona pellucida* fracturing which is common in conventionally frozen embryos. Its frequency varied around 27% in embryos vitrified in sealed straws, but did not exceed 1% in those vitrified in open capillaries (Vajta et al., 1999). Our data are consistent with those of Lazar et al. (2000) who observed this damage only sporadically.

In vitro produced embryos are known to be very sensitive to freezing by the conventional procedure (Leibo et al., 1996; Seidel Jr., 2006; Vajta and Kuwayama, 2006) because this culture system can affect cryotolerance and consequently the quality of blastocysts (Rizos et al., 2001). It is possible to increase post-thaw survival of vitrified IVP embryos by supplementing growth factors and growth hormone in the culture media (Mtango et al., 2003). Removal of cytoplasmic lipids has improved survival but it made the process technically very demanding (Vajta and Kuwayama, 2006). Reduction of cytoplasmic lipid contents of *in vitro* produced embryos with phenazine ethosulfate (PES), a compound that oxidized NADPH, even improved cryotolerance of bovine embryos cultured in the absence of serum (Seidel Jr., 2006).

Lewis et al. (1999) studied pregnancy rates after transfer of *in vitro* produced embryos vitrified in OPS. The embryos were thawed either directly by transfer into 0.25-ml straws containing the holding medium with 0.2 mol/l sucrose, or outside of straw. In the former group, 22 embryos were transferred to 11 recipients of which 7 (64%) became pregnant with a total of 11 foetuses, i.e. 50% of the total number of the transferred embryos. In the latter group, 20 embryos were transferred to 10 cows of which 4 (40%) became pregnant with 7 foetuses. Lazar et al. (2000), who transferred 18 vitrified embryos into 6 recipients reported 50% pregnancy rate.

This study was focused on pregnancy rates after transfer of *in vivo* produced bovine embryos of various quality grades and developmental stages which were cryopreserved by vitrification in OPS or by conventional freezing. Only one embryo was transferred to each recipient. No difference was found between the conception rates after transfer of embryos at the stage of morula (54.5% and 56.0%) or early blastocyst (53.3% and 58.1%). Somewhat lower values of pregnancy rate were obtained after transfer of expanded blastocysts cryopreserved by conventional freezing (48.3%) in comparison with embryos cryopreserved by vitrification in OPS (57.7%). But the difference was not significant ($P > 0.05$). Our previous study (Lopatarova et al., 2002) demonstrated higher survival rate of embryos in advanced developmental stages (expanded blastocyst) which were cryopreserved by OPS vitrification compared to embryos cryopreserved conventionally ($P < 0.05$) in the culture conditions. Riha (1994) transferred intact *in vivo* produced bovine embryos (day seven) after one step vitrification method. Conception rate of vitrified embryos washed in 1M sucrose in culture medium was lower (27.9%) than after washing in 0.8M sucrose (56.7%) or 0.5M sucrose (50%). The conception rate in the control group (routine one step method) was 60%. Riha et al. (2002) evaluated pregnancy rates after transfer of cryopreserved bovine embryo produced *in vivo* and *in vitro*. In addition they tested various cryoprotectants. The values of pregnancy rate after transfer of *in vivo* produced frozen/thawed embryos were 66.2% (embryos preserved by glycerol

procedure), 53.1% (embryos preserved by ethylene glycol procedure) and 64.3% (embryos vitrified by one step method) while different conception rates were after transfer of *in vitro* produced embryos: 34.7% (glycerol procedure), 72% (ethylene glycol procedure) and 50% (one step vitrification).

No difference was found between the conception rates after transfer of vitrified embryos at the stage of morula (54.5%), early blastocyst (53.3%) and expanded blastocyst (57.7%) in our study. In contrast Ravindranatha et al. (2001) showed that pregnancy rate after embryo transfer varied significantly according to the stages of embryo development. The pregnancy rates following *in vivo* produced vitrified compact morulae, early blastocysts, blastocysts and expanded blastocysts regardless of quality grades were 25%, 37.5%, 50% and 0%, respectively. On the other hand pregnancy rates following transfer of programmable frozen compact morulae, early blastocysts, blastocysts and expanded blastocysts regardless of quality grades were 29%, 50%, 33% and 50%, respectively. Analogous to our observations, the studies of Vajta et al. (1997b, 1998a, 1999) and Lazar et al. (2000) demonstrated that advanced stages of *in vitro* produced bovine embryos can be vitrified successfully with hatching rates reaching 60% to 94%. Even *in vitro* produced embryos vitrified at the hatched blastocyst stage reached up to 81% re-expansion rate after 24 h of culture. In the study of Riha and Vejnar (2004) expanded, hatching or hatched porcine blastocysts were collected *post mortem* from superovulated donors in days 5.5 and 6 after insemination. The embryos were vitrified, thawed and transferred into synchronized recipients (15 embryos per recipient). The pregnancy rates were 57.3% or 67.0% (two vitrification protocols) and 42.7% in controls (transfer of fresh embryos).

The quality of development of the early embryo determines the quality of the blastocyst as well as the final results. In our study, the transfer of quality Grade 1 embryos after thawing demonstrated similar survival rates (55.1% and 54.1%) following both freezing methods. Lower pregnancy rates we found after transfer of quality Grade 2 embryos (36.4% in vitrified embryos and 32.9% in conventionally frozen embryos). Van der Zwalmen et al. (2003) also observed that human blastocysts of optimal quality had survival rates, implantation rates and ongoing pregnancy rates 73%, 32% and 19%, respectively. In contrast, after transfer of blastocysts of sub-optimal quality values of those parameters were only 38%, 9% and 6%.

The risk of contamination, that may result from direct contact of the straw content with liquid nitrogen, can be eliminated by nitrogen filtration (Vajta et al., 1998b) or freezing in concentrated nitrogen vapour (–170°C), and covering open ends of the straw with a sealed protective straw. Another method that can considerably reduce the contamination is repeated washing of thawed embryos in several drops of the culture medium.

A one step vitrification procedure for bovine embryos (day 7) obtained by superovulation and transferred to recipients directly without post-thawing morphological control was developed by Riha (1994). Transfer of embryos performed on day 7 after oestrus synchronization resulted in lower conception rate (45%) compared to transfer after spontaneous oestrus (63.6%). Direct transfer and sterile performance of the open pulled straw vitrification method is also possible (Vajta et al., 1998b, Vajta, 2003).

On the basis of our results it can be concluded that vitrification can simplify, accelerate and provide a more cost efficient cryopreservation of *in vivo* produced bovine embryos without negative effect on survival rate after transfer into recipients.

REFERENCES

Abe Y., Hara K., Matsumoto M., Kobayashi J., Sasada H., Ekwall H., Rodriguez-Martinez M., Sato E. (2005): Feasibility of nylon-mesh holder for vitrification of bovine germinal vesicle oocytes in subsequent production of viable blastocysts. Biology of Reproduction, 72, 1416–1420.

Arav A., Zeron Y., Ocheretny A. (2000): A new device and method for vitrification increases the cooling rate and allows successful cryopreservation of bovine oocytes. Theriogenology, 53, 248.

Asada M., Ishibashi S., Ikumi S., Fukui Y. (2002) Effect of polyvinyl alcohol (PVA) concentration during vitrification of *in vitro* matured bovine oocytes. Theriogenology, 58, 1199–1208.

Baguisi A., Lonergan P., Overstrom E.W., Boland M.P. (1999): Vitrification of bovine embryos: Incidence of necrosis and apoptosis. Theriogenology, 51, 62.

Celestinos M., Gatica R. (2002): Vitrification as a technique of bovine embryo cryopreservation. Archivos de Medicina Veterinaria, 34, 157–165.

Dattena M., Ptak G., Loi P., Cappai P. (2000): Survival and viability of vitrified *in vitro* and *in vitro* produced ovine blastocysts. Theriogenology, 53, 1511–1519.

Holy L., Lopatarova M., Krontorad P., Holy J. (1990): Systematic use of embryo transfer in cattle under condition of high animal concentration (in German). Monatsheft Veterinaermedizin, 45, 409–413.

Hurtt A.E., Landim-Alvarenga F., Seidel Jr. G.E., Squires E. L. (2000): Vitrification of immature and mature equine and bovine oocytes in an ethylene glycol, ficoll and sucrose solution using open pulled straws. Theriogenology, 54, 119–129.

Kong I.K., Lee S.I., Im Y.T., CHo S.G.,Ohh H.J., Ohh D.H., Bae I.H. (2000): Improvement of post-thaw hatching rates of *in vitro* produced bovine embryos vitrified by ultra-mini straw. Theriogenology, 53, 258.

Lazar L., Spak J., David V. (2000): The vitrification of *in vitro* fertilized cow blastocyst by the open pulled straw method. Theriogenology, 54, 571–578.

Leibo S.P., Loskutoff N.M. (1993): Cryobiology of *in vitro*-derived bovine embryos. Theriogenology, 39, 81–94.

Leibo S.P., Martino A., Kobayashi S., Pollard J.W. (1996): Stage dependent sensitivity of oocytes and embryos to low temperatures. Animal Reproduction Science, 42, 45–53.

Lewis I.M., Lane M.W., Vajta G. (1999): Pregnancy rates following transfer of *in vitro* produced bovine embryos vitrified by the open pulled straw (OPS) method. Theriogenology, 51, 168.

Lopatarova M., Cech S., Havlicek V., Holy L. (2002): Effect of vitrification in open pulled straws on survival of bovine embryos from superovulated cows. Acta Veterinaria Brunensis, 71, 93–99.

Massip A., Van der Zwalmen P., Ectors F. (1987): Recent progress in cryopreservation of cattle embryos. Theriogenology, 27, 69–79.

Massip A., Van der Zwalmen P., Scheffen B., Ectors F. (1989): Some significant steps in the cryopreservation of mammalian embryos with a note on a vitrification procedure. Animal Reproduction Science, 19, 117–129.

Matsuoka K., Sakata S., Ichino K., Shimaya S., Katagihara T., Suzuki T. (1995): Ultra-rapid freezing of *in vitro* produced bovine embryos. Theriogenology, 43, 274.

Merton J.S., Oei C., Otter Z., Haring R., Vajta G. (2001): Effect of cryopreservation method (glycerol, ethylene glycol and open pulled straw) on *in vitro* survival of slaughterhouse and OPU derived IVP embryos. Theriogenology, 55, 312.

Mtango N.R., Varisanga M.D., Dong Y.J., Rajamahendran R., Suzuki T. (2003): Growth factors and growth hormone enhance *in vitro* embryo production and post-thaw survival of vitrified bovine blastocysts. Theriogenology, 59, 1393–1402.

Papis K., Shimitzu M., Izaike Y. (2000): Factors affecting survivability of bovine oocytes vitrified in droplets. Theriogenology, 54, 651–658.

Ravindranatha B.M., Nandi S., Reddy S.M. (2001): Vitrification and programmable freezing of different stages of bovine embryos – pregnancy and calving following transfer. Indian Journal of Dairy Science, 54, 75–79.

Riha J. (1994): One-step vitrification method for cryopreservation of bovine embryos transferred into recipients. Reproduction in Domestic Animals, 29, 46–54.

Riha J., Vejnar J. (2004): Comparison of two vitrification methods for cryopreservation of porcine embryos. Czech Journal of Animal Science, 49, 183–189.

Riha J., Machatkova M., Pavlok A. (2002): Viability of fresh and frozen transferred IVP bovine embryos. Czech Journal of Animal Science, 47, 261–267.

Rizos D., Ward F., Boland M.P., Lonergan P. (2001): Effect of culture system on the yield and duality of bovine blastocysts as assessed by survival after vitrification. Theriogenology, 56, 1–16.

Schiewe M.C. (1991): The science and significance of embryo cryopreservation. Journal Zoo and Wildlife Medicine, 22, 6–22.

Seidel Jr. G.E. (2006): Modifying of oocytes and embryos to improve their cryopreservation. Theriogenology, 65, 228–235.

Shaw J.M., Oranratnachai A., Trounson A.O. (2000): Fundamental cryobiology of mammalian oocytes and ovarial tissue. Theriogenology, 53, 59–72.

Vajta G. (2003): Vitrification of domestic animal embryos. In: Proceedings from the 5th World Conference of A-PART (The International Association of Private Assisted Reproductive Technology Clinics and Laboratories), 23–25 October 2003, Tokyo, Japan, 133–138.

Vajta G., Kuwayama M. (2006): Improving cryopreservation systems. Theriogenology, 65, 236–244.

Vajta G., Booth P.J., Holm P., Greve T., Callesen H. (1997a): 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., Greve T., Callesen H. (1997b): Survival and development of bovine blastocysts produced *in vitro* after assisted hatching, vitrification and in-straw direct rehydration. Journal of Reproduction and Fertility, 111, 65–70.

Vajta G., Holm P., Greve T., Callesen H. (1997c): Vitrification of porcine embryos using the open pulled straw (OPS) method. Acta Veterinaria Scandinavica, 38, 349–352.

Vajta G., Holm P., Kuwayama M., Booth P.J., Jacobsen H., Greve T., Callesen H. (1998a): Open pulled straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. Molecular Reproduction and Development, 51, 53–58.

Vajta G., Lewis I.M., Kuwayama M., Greve T., Callesen H. (1998b): Sterile application of the open pulled straw (OPS) vitrification method. Cryo-Letters, 19, 389–392.

Vajta G., Rindom N., Peura T.T., Holm P., Greve T., Callesen H. (1999): The effect of media, serum, and temperature on *in vitro* survival of bovine blastocysts after open pulled straw (OPS) vitrification. Theriogenology, 52, 939–948.

Van der Zvalmen P., Bollen N., Lejeune B., Schoysman R., Zech H., Zech N. (2003): Rapid and ultra-rapid vitrification procedures: Factors affecting the results. In: Proceedings from the 5th World Conference of A-PART (The International Association of Private Assisted Reproductive Technology Clinics and Laboratories), 23–25 October 2003, Tokyo, Japan, 122–132.

Voelkel S.A., Hu Y.X. (1992): Direct transfer of frozen-thawed bovine embryos. Theriogenology, 37, 23–37.

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