Effect of Vitrification in Open Pulled Straws on Survival of Bovine Embryos from Superovulated Cows

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Abstract

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Bovine embryos (Day $_{6.5-7.5}$) collected from superovulated cows were exposed to open pulled straw (OPS) vitrification before *in vitro* culture for 72 hours. The aim of this cryopreservation study applied to embryos of different developmental stages and morphological quality grades was to assess embryo survival and process of embryo handling during commercial embryo transfer (ET) procedures using the conventional freezing method as a control.

During the culture of thawed quality grade 1 embryos, the hatched blastocyst stage was reached by 38.6% (27/70) of the vitrified and by 34.5% (29/84) of the conventionally frozen (control) embryos ($p > 0.05$). The corresponding proportions for quality grade 2 and 3 embryos were 18.5% (10/54) vs. 5.4% (2/37) and 11.8% (8/68) vs. 2% (1/50), $p > 0.05$, respectively.

Hatching rates of embryos vitrified or conventionally frozen at the morula stage were 35.7% (15/42) and 30.3% (17/56), $p > 0.05$, respectively. No significant difference was found between hatching rates of embryos vitrified or conventionally frozen at the stage of early blastocysts (29.3%; 12/41 vs. 34%; 18/54). The hatching rates of embryos vitrified or conventionally frozen at the blastocyst and expanded blastocyst stages were 30.8% (12/39) vs. 21.6% (11/51), $p > 0.05$ and 29.3% (11/37) vs.11.4% (5/44), $p < 0.05$, respectively.

The study demonstrated about the same survival rates for vitrified and conventionally cryopreserved embryos of all quality grades and developmental stages during *in vitro* embryo culture. Expanded blastocysts survived better vitrification than conventional freezing ($p < 0.05$). OPS vitrification is an effective and rapid method of cryopreservation of bovine embryos.

Bovine embryo, superovulation, cryopreservation, freezing, thawing, vitrification, OPS vitrification

Cryopreservation of embryos has become a widely used method in commercial embryo transfer for many reasons as evidenced by the increasing number of cryopreserved embryos as well as their survival quality, which reaches nearly the survival rate of fresh embryos (Shaw et al. 2000).

Conventional cryopreservation is a slow procedure which exposes the embryo at various phases of freezing to the action of a range of physical, chemical and biological factors. This action can result in disruption of *zona pellucida*, cell membranes, and cytoskeleton, and the ensuing metabolic disturbances. Such cell damage leads to a loss of cell self-control and eventually to cell death by apoptosis or necrosis (Baguisi et al. 1999). The highest danger for embryonic cells is posed by intracellular crystalline ice formation (Massip et al. 1989; Schiewe 1991; Leibo and Loskutoff 1993; Matsuoka et al. 1995; and others) that can occur under specific conditions of freezing and thawing and affects recuperation and survival of embryonic cells. Moreover, the slow cryopreservation procedure requires a sophisticated and expensive equipment.

Vitrification has been known as an alternative to conventional methods of cryopreservation for a long time. The first to use it for freezing of mammalian embryos were

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Rall and Fahy (1985) and later Massip et al. (1986; 1989). This procedure 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 of it, the viscosity of the cryoprotectant increases to form a solid glass-like mass.

Vitrification of embryos can be carried out in 0.25-mL straws (Massip et al. 1986; Ishimori et al. 1993; Říha 1994; Lane et al. 1998; Hurtt et al. 2000; and others), in glass micropipettes (Kong et al. 2000a) or in microdrops of the cryoprotectant solution (Říha et al. 1991; Yang and Leibo 1999; Papis et al. 1999; 2000).

Vitrification considerably simplifies and accelerates the cryopreservation process without requiring expensive equipment. It has also been found suitable for cryopreservation of oocytes (Vajta et al. 1998; Hurtt et al. 2000; Araw et al. 2000; Papis et al. 2000) and particularly of *in vitro* produced embryos, which are highly sensitive to freezing owing to their high content of lipids (Vajta 1997; Vajta et al. 1997ab; 1998; 1999, Lewis et al. 1999; Dattena et al. 2000; Kong et al. 2000b; Lazár et al. 2000; Merton et al. 2001). Vitrification has also been found suitable for freezing of advanced stages of embryos, including *in vivo* or *in vitro* produced hatched blastocysts, where the conventional cryopreservation method would be fatal (Vajta et al. 1997b).

A significant step forward in the use of cryopreservation has been vitrification in open pulled straws (OPS). Its benefits include minimisation of the volume of the cryoprotectant solution in the narrowed 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 1997; Vajta et al. 1997a, 1998, 1999; Lewis et al. 1999; Dattena et al. 2000; Lazár et al. 2000; and others). The rapid freezing prevents the formation of crystalline ice and zona fracturing; toxic and osmotic effects at thawing are minimised by immersion of the embryo-containing capillary into a thawing solution.

The objective of our study, in which results of vitrification of embryos in OPS were compared with those of conventional freezing, was to find whether vitrification method is suitable for cryopreservation of advanced stages of *in vivo* produced embryos as well as low-quality embryos which are less suitable for cryopreservation by the conventional method.

Materials and Methods

Animals and treatment

Healthy donor cows were superovulated and their uteri were flushed for embryo collection. All the cows were superovulated in the luteal phase (between days 8-12) 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 treatment (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 luteolysis.

Embryo collection and scoring

Seven days after oestrus, the 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). The flushing as well as embryo isolation were performed as described by Holý et al. (1990).

The obtained embryos were washed with PBS + 10% FCS and evaluated for developmental stage and quality using morphological criteria. Morphologically intact compacted morulae, early blastocysts, blastocyst and expanded blastocyst of quality 1 (morphologically intact, even granulation and cell distribution), quality 2 (small deviations, e.g. few excluded blastomeres), and quality 3 (uneven cell organization, loosened structure with numerous free blastomeres) were cryopreserved either by OPS vitrification or conventional freezing, and subsequently cultured *in vitro*.

Vitrification

Vitrification was done as described by Vajta et al. (1998). 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 wall thickness of 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 first 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 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 sec.

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.

Embryo thawing and culture

Thawing of vitrified embryos was performed by expelling the embryos contained at 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 ºC in all the steps. Subsequently, the vitrified and the conventionally frozen/thawed (control) embryos were transferred into E 199 (Parker's medium with Earle's salts, Sevapharma Prague, Czech Republic) supplemented with 545 µg/mL Lactic Acid-Hemicalcium Salt (Sigma-Aldrich Chemie, Steinheim, Germany), 90 µg/mL L-Glutamine (Sigma-Aldrich Chemie, Steinheim, Germany), 2.727 mg/mL Sodium Bicarbonate (Sigma-Aldrich Chemie, Steinheim, Germany), 1.273 mg/mL Hepes (Serva, Heidelberg, Germany), 227 µg/mL Pyruvic acid (Sigma-Aldrich Chemie, Steinheim, Germany), 1µg/mL Gentamycin (Sigma-Aldrich Chemie, Steinheim, Germany) and 10% FCS. The embryos were cultured in groups in 4-well culture dishes under mineral oil (Sigma-Aldrich Chemie, Steinheim, Germany) at 39 ºC in an atmosphere of 5% CO_2 in humid air for 72 h. Checks for developmental stage were done at 24-h intervals.

Statistical analysis

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

Results

The results of *in vitro* embryo survival in terms of morphological quality are shown in Table 1. The survival and hatching rates of vitrified quality grade 1 embryos were 92.9% (65/70) and 52.9% (37/70), respectively. The corresponding rates for the control embryos were 91.7% (77/84) and only 53.6 % (45/84), $p > 0.05$. Complete hatching was observed in 38.6% (27/70) of the vitrified and in 34.5% (29/84) of the control embryos ($p > 0.05$). Survival rates of vitrified quality grade 2 and 3 embryos were 77.4% (48/62) and 55.1% (27/49), respectively and the corresponding rates for the control embryos were 63.2% (43/68) and 42% (21/50), respectively. The hatching blastocyst stage was reached by 35.5% (48/62) and 22.4% of the vitrified embryos of quality grade 2 and 3, respectively, and by 26.5% (18/68) and 12% (6/50), $p > 0.05$ of conventionally frozen embryos of quality grades 2 and 3, respectively. Complete hatching was reached by 22.6% (14/62) and 10.2% (5/49) of the vitrified and by 11.8% (8/68) and 2% (1/50) of the conventionally frozen embryos of the respective quality grades ($p > 0.05$).

Table 1
In vitro development of bovine embryos ($D_{6.5\text{-}7.5}$) of different quality after OPS vitrification

Quality grade	Freezing	No embryos cultured	No (%) embryos developing		
			in culture	to hatching blastocyst	to hatched blastocyst
1	Vitrification	70	65 (92.9)	37 (52.9) a	27 (38.6) c
	Conventional	84	77 (91.7)	45 (53.6) b	29 (34.5) d
2	Vitrification	62	48 (77.4)	22 (35.5) e	14 (22.6) g
	Conventional	68	43 (63.2)	18 (26.5) f	8 (11.8) h
3	Vitrification	49	27 (55.1)	11 (22.4) i	5 (10.2) k
	Conventional	50	21 (42)	6 (12) j	1 (2) l

a:b, c:d, e:f, g:h, i:j, k:l non-significant ($p > 0.05$)

Table 2 summarizes *in vitro* survival rates of embryos frozen at different stages of development. The survival, hatching, and complete hatching rates of embryos vitrified or conventionally frozen at the morula stage were 90.5% (38/42) vs. 92.9% (52/56), 52.4% (22/42) vs. 46.4% (26/56), and 35.7% (15/42) vs. 30.3% (17/56), respectively ($p > 0.05$).

Table 2
In vitro development of bovine embryos ($D_{6.5 - 7.5}$) of different stage after OPS vitrification

Stage of development	Freezing	№ embryos cultured	№ (%) embryos developing		
			in culture	to hatching blastocyst	to hatched blastocyst
Morula	Vitrification	42	38 (90.5)	22 (52.4) a	15 (35.7) c
	Conventional	56	52 (92.9)	26 (46.4) b	17 (30.3) d
Early blastocyst	Vitrification	41	37 (90.2)	20 (48.8) e	12 (29.3) g
	Conventional	53	49 (92.5)	27 (50.9) f	18 (34) h
Blastocyst	Vitrification	39	33 (84.5)	21 (53.8) i	12 (30.8) k
	Conventional	51	44 (86.3)	23 (45.5) j	11 (21.6) l
Expanded blastocyst	Vitrification	37	30 (81.1)	15 (40.5) m	11 (29.3) o
	Conventional	44	32 (72.7)	10 (22.7) n	5 (11.4) p

a:b, c:d, e:f, g:h, i:j, k:l,m:n non-significant ($p > 0.05$)
o:p $p < 0.05$

The survival, hatching, and complete hatching rates of embryos vitrified or conventionally frozen at the early blastocyst stage were 90.2% (37/41) vs. 92.5% (49/53), 48.8% (20/41) vs. 50.9% (27/43), and 29.3% (12/41) vs. 34% (18/53), respectively ($p > 0.05$).

The survival, hatching, and complete hatching rates of embryos vitrified or conventionally frozen at the blastocyst stage were 84.5% (33/39) vs. 86.3% (44/51), 53.8% (21/39) vs. 45.5% (23/51), and 30.8% (12/39) vs. 21.6% (11/51), respectively ($p > 0.05$).

The survival, hatching, and complete hatching rates of embryos vitrified or conventionally frozen at the expanded blastocyst stage were 81.8% (30/37) vs. 72.7% (32/44), 40.5% (15/37) vs. 22.7% (10/44), and 29.3% (11/37) vs. 11.54% (5/44), respectively. Only the latter difference was significant ($p > 0.05$).

Discussion

Successful use of vitrification in OPS results from a considerable thinning of the straw wall from 1.7 to 0.8 mm, which markedly reduces the thermal insulation effect, and from a considerable reduction of the inner diameter of OPS from 0.85 to 0.07 mm, which minimises the volume of the cryoprotectant and enhances capillary lift of the embryo-containing cryoprotectant solution maintaining its constant level in the capillary. The temperature drop from 0 ºC to -196 °C is accelerated by direct contact of the cryoprotectant solution with liquid nitrogen to 16 700 - 22 500 ºC per min, which is a tenfold of the freezing speed in sealed 0.25-mL straws. The efficacy of the OPS vitrification is further enhanced by the direct contact of the embryos with the thawing solution and minimisation of exposure to its toxic and other adverse effects on the embryo. Rehydration of embryo occurs immediately after immersion into the thawing solution (Vajta et al. 1997a; 1998).

Another benefit of OPS is a significant reduction or complete elimination of zona 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 Lazár et al. (2000) who observed this damage only sporadically.

Recently Kong et al. (2000a, b) have suggested that glass capillaries pulled to an outer diameter of 0.3 mm can also be used in the open vitrification method. Their advantage consists in a much higher thermal conductivity of glass which accelerates the freezing and thawing processes.

The risk of contamination, that may result from the direct contact of the straw content with liquid nitrogen, can be eliminated by nitrogen filtration or freezing in concentrated nitrogen vapour (-170 ºC) and subsequent covering of the 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.

In vitro produced embryos as well as oocytes are known to be very sensitive to freezing by the conventional procedure (Leibo et al. 1996) and the same applies to embryos at advanced stages of development (blastocysts, expanded and hatched blastocysts). Less suitable or unsuitable for freezing are also embryos showing severe morphological defects or low embryonic cell numbers that were collected from superovulated cows.

The studies of Vajta et al. (1997b; 1998; 1999) and Lazár et al. (2000) have demonstrated that advanced stages of *in vitro* produced embryos can be vitrified successfully with hatching rates reaching 60 to 94%. Even *in vitro* produced embryos vitrified at the hatched blastocyst stage reached an up to 81% re-expansion rate after 24 h of culture.

Vitrification was also survived by partly denuded oocytes frozen 6 to 24 h after collection and fertilized after 24 h of maturation. Their cleavage rates varied between 47 and 72%, as compared with 87% in controls, and the blastocyst stage was reached by 7 to 13% of them (Vajta et al. 1998).

Lewis et al. (1999) studied pregnancy rates after transfer of *in vitro* produced embryos vitrified in OPS. The embryos were thawed either directly by transferring them into 0.25-mL straws containing the holding medium with 0.2 mol/L sucrose, or outside the straws. 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. Lazár et al. (2000), who transferred 18 vitrified embryos into 6 recipients reported 50% pregnancy rate.

This study was focused on survival rates of *in vitro* produced bovine embryos of various quality grades and developmental stages as influenced by vitrification in OPS or conventional freezing. In quality grade 1 embryos, the culture after thawing demonstrated a survival rate of 92% to 93% irrespective of the freezing method used (Table 1). The hatched blastocyst stage was reached by 38.6% of the vitrified and 34.5% of the conventionally frozen embryos.

Continuing development of quality grade 2 embryos was observed in 77.4% of the vitrified and in 63 % of the conventionally frozen embryos and the blastocyst stage was reached by 22.6% of the vitrified embryos and 11.8% of the conventionally frozen embryos. The survival rates of quality grade 3 embryos after vitrification and conventional freezing were 55.1% and 42.0%, respectively. However, only 10.2% of the vitrified and 2.0% of the conventionally frozen embryos developed up to the stage of hatched blastocysts.

The survival rates of embryos after OPS vitrification or conventional freezing at the stage of morula were rather high reaching 90% to 93%. Somewhat lower values were obtained for

embryos frozen conventionally at the stage of blastocysts (86%) and hatched blastocysts (72%). Survival up to the hatched blastocyst stage was found in 35.7%, 29.3%, 30.8%, and 29.3% of the embryos vitrified at the morula, early blastocyst, blastocyst, and expanded blastocyst stages, respectively. The corresponding values for conventional freezing were 30.3% for morulae, 34% for early blastocysts, 21.6% for blastocysts and only 11.4% for expanded blastocysts. Compared with the effect of vitrification, the latter difference was significant at $p < 0.05$.

It can be concluded from the results presented here that vitrification can improve, simplify and accelerate the process of cryopreservation of embryos. Another benefit of vitrification is that no expensive equipment is necessary.

Přežívání embryí skotu po vitrifikaci v otevřených pejetách

Embrya skotu získaná superovulací ve stáří 6.5 – 7.5 dnů byla zmrazována vitrifikační metodou v otevřených pejetách (open pulled straw, OPS) a po rozmrazení kultivována *in vitro* po dobu 72 hod. Vitrifikační postup byl aplikován u embryí různých vývojových kategorií a rozdílné morfologické kvality a výsledky přežívání byly srovnány s přežíváním embryí zmrazených konvenční metodou používanou v rámci komerčního embryo transferu.

Během kultivace morfologicky dobrých (kvalitativní stupeň 1) zárodků dosáhlo stádia vyklubané blastocysty 38.6% (27/70) vitrifikovaných a 34.5% (29/84) konvenčně zmrazených kontrolních embryí ($p > 0.05$). Vitrifikovaná embrya horších kvalit (kvalitativní stupeň 2 a 3) dosáhla stádia vyklubané blastocysty v 18.5% (10/54) resp. 5.4% (2/37), zatímco konvenčně zmrazená embrya se vyklubala jen v 11.8% (8/68) resp. 2% (1/50) případů ($p > 0.05$).

Vitrifikovaná embrya ve stádiu moruly se po rozmrazení vyvíjela do vyklubané blastocysty ve 35.7% (15/42), zatímco konvenčně zmrazené-rozmrazené moruly dosáhly vyklubání ve 30.3% (17/56) případů ($p > 0.05$). Nesignifikantní rozdíl v úrovni vyklubání - 29.3% (12/41) a 34% (18/54) - byl zaznamenán při kultivaci ve skupinách vitrifikovaných a rutinně zmrazených/rozmrazených časných blastocyst. Vitrifikované blastocysty se vyklubaly ve 30.8% (12/39), kontrolní zárodky jen ve 21.6% (11/51) případů ($p > 0.05$). Úspěšné vyklubání vitrifikovaných expandovaných blastocyst bylo registrováno ve 29.3% (11/37), u kontrolních embryí však pouze v 11.4% (5/44) případů ($p < 0.05$).

Dosažené výsledky ukazují srovnatelnou úroveň přežívání vitrifikovaných a konvenčně zmrazených/rozmrazených embryí všech morfologických kategorií a vývojových stádií během kultivace *in vitro*. Expandované blastocysty přežily vitrifikační postup lépe ($p < 0.05$) než klasické zmrazování. Vitrifikační metodu v otevřených pejetách lze považovat za efektivní a rychlou cestu pro kryokonzervaci embryí skotu.

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