Comparison of two vitrification methods for cryopreservation of porcine embryos

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ABSTRACT: The aim of this study was to compare two vitrification methods of porcine perihatching blastocysts with regard to the success of transfer of these embryos to the recipients. Expanded, hatching, or hatched blastocysts were recovered post mortem from superovulated donors in 5.5 to 6.0 days after artificial insemination of donor gilts with homospermic doses. In protocol VS I, the embryos in perihatching developmental stage were equilibrated in a culture medium H-MEMD with 10% v/v of glycerol (1.37M solution of glycerol in medium) for 10 min and placed in a vitrification medium for 1.5 min max. (vitrification medium contained 50% v/v 2M sucrose in tridistilled water, 30% v/v of glycerol, and 20% v/v of foetal calf serum – FCS). Then they were dropped with micropipette and stored in liquid nitrogen vapour. For protocol VS II, we used H-MEMD culture medium supplemented with 20% v/v of FCS, 25% v/v ethylene glycol, and 25% v/v dimethyl sulphoxide (DMSO). Embryos were equilibrated for 10 min in a mixture of the vitrification medium and culture medium (1 : 1), and were kept in the vitrification medium for 1.5 minutes. Then they were dropped with micropipette and stored in liquid nitrogen vapour. Embryos were thawed by immersing the drop with the embryo in H-MEMD culture medium with 0.8M sucrose for 10 minutes. After thawing and washing in the medium with sucrose, all embryos were washed three times in a fresh medium and prepared for transfer. Recipients were synchronized either using Regumate-feeding followed by treatment with PMSG and HCG (gilts) or using piglet weaning (sows – 1st and 2nd parity). Recipients showing standing heat at the time of donor insemination were used for laparoscopic and non-surgical ET on day 5.5–6.0 of the cycle. The fraction of viable embryo vitrified under VS I or VS II protocol was 85% and 80%, compared to 95% in control fresh embryos ($P > 0.05$). Pregnancy of recipients was 57.3% (5/7), 67.0% (4/6) for VS I or VS II group and 42.7% (10/23) for control ($P < 0.001$). We can conclude on the basis of our data that both protocols for vitrification yielded similar results and can be used for cryopreservation of porcine embryos.

Keywords: pig; embryo; transfer; cryopreservation; vitrification; recipient; gilt; sow; natality

Embryo transfer is applied routinely in reproduction and selection in cattle, sheep, and goats. However, cryopreservation of pig embryos has encountered numerous problems stemming from the specific features of porcine embryos and from the specific features in physiology and morphology of sow's reproductive organs.

Porcine embryos are highly sensitive to chilling and cryopreservation is very difficult (Polge *et al*., 1974; Pollard and Leibo, 1994). Therefore the cryopreservation of porcine embryos has not proceeded beyond the experimental stage (Niemann, 1991; Brussow and König, 1992). Success has been confined mainly to the cryopreservation of embryos at the stage of perihatching blastocysts – Fujino *et al*. (1993), Nagashima *et al*. (1995), Kobayashi *et al*. (1998), Dobrinsky *et al*. (1999) reported successful cryopreservation of embryos at the stage of compacted morulae or early blastocysts after removing cytoplasmic lipid droplets using micro-

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manipulation according to the method described by Nagashima *et al*. (1995). Even our studies (Říha *et al*., 1996, 1997a,b; Říha and Vejnar, 2000, 2003) indicated that in spite of positive and favourable results, numerous problems still persist in cryopreservation of porcine embryos.

Surgical procedures of embryo recovery and embryo transfer (ET) are also the significant limiting factors for commercial utilization of ET in swine. The non-surgical transfer of porcine embryos has encountered very limited success from its early days (Polge and Day, 1968). The anatomical structure of the cervix and the small orifice of the cervical channel complicate the penetration of a catheter into the uterus lumen. Despite some success in non-surgical methods of transcervical transfers of fresh porcine embryos (Reichenbach *et al*., 1993; Galvin *et al*., 1994; Modl, 1994; Hazeleger *et al*., 1995a,b; Wallenhorst and Holz, 1995) the methods for porcine ET are still in the experimental phase of their development.

The aim of the present study was to compare two vitrification protocols for cryopreservation of perihatched porcine embryos. For these purposes, we vitrified pig embryos and checked their viability after *in vitro* culture or after surgical or non-surgical transfer to recipient sows.

MATERIAL AND METHOD

Animals

Donors. Pubertal selected breeding gilts (Czech super meat and Landrace) superovulated according to the schemes described by Říha and Vejnar (2000) were used as embryo donors (feeding of Regumate for 15 days and treatment with 750 I.U. PMSG and 300 I.U. HCG). Homospermic doses were used for insemination.

Recipients. Sows after 1st or 2nd parturition were synchronized using the weaning of piglets between Day 28 to Day 32 *post partum*. Pubertal gilt-recipients were synchronized using Regumate (active agent: altrenogest, producer: Hoechst, doses recommended by the producer). They were fed Regumate for 15 days and then treated with 500 I.U. PMSG (Sergon, ad us. vet., Bioveta Ivanovice in Haná, CR) and 300 I.U. HCG (Praedyn, Léčiva Prague, C.R., or Werfachor, Austria). Recipients showing the standing heat at the time of donor insemination were used for ET only.

Embryo cryopreservation methods

Two different vitrification protocols were used for cryopreservation of porcine embryos.

Protocol VS I. The embryos in perihatching developmental stage were equilibrated in a culture medium H-MEMD with 10% v/v of glycerol (1.37M solution of glycerol in medium) for 10 minutes. Then they were placed in a vitrification medium for 1.5 min max. (the vitrification medium contained 50% v/v 2M sucrose in tridistilled water, 30% v/v of glycerol, and 20% v/v of foetal calf serum – FCS). Finally, the embryos were dropped with micropipette and stored in liquid nitrogen vapour. Embryos were thawed after the immersion of drop with embryo in a medium containing 0.8 M sucrose for 10 minutes.

Protocol VS II. For this protocol, we used a medium composed of 25% v/v ethylene glycol + 25% v/v dimethyl sulphoxide (DMSO) in H-MEMD culture medium supplemented with 20% v/v of FCS. Embryos were equilibrated for 10 min in a mixture of the vitrification medium and culture medium (1 : 1) and were kept in a vitrification medium for 1.5 minutes. Finally they were dropped with a micropipette and stored in liquid nitrogen vapour. For thawing, embryos were placed in a medium with 0.8M sucrose in H-MEMD culture medium.

After thawing and washing in the medium with sucrose, embryos were washed three times in a fresh medium and prepared for transfer. All manipulations with embryos were done at laboratory temperature.

Embryo long-term culture. To evaluate the quality of vitrified embryos after thawing, we cultured thawed embryos for 36 h in a culture medium H-MEMD with 20% (v/v) of FCS in 0.5 ml closed straw. Hatched embryos or embryos with reexpanded blastocoel, undistorted trophoblast, and compact embryoblast were classified as viable (Říha *et al*., 1997a).

Embryo recovery, short-term culture, and transfer. *Post mortem* recovery of embryos from donors, embryo culture, their morphological classification, and laparoscopic transfer of embryos were performed as described previously (Říha *et al*., 1996, 1997a,b; Říha and Vejnar, 2000, 2003). The non-surgical intubation (transfer) was made with an A.I. probe for intracervical insemination. A silicone (adequately rigid) catheter fitted to the syringe containing the culture medium (5 ml approx.) was

drawn through the probe. We preferred using sows of different colours for donors and recipients.

Transferred embryos. Fresh embryos stored in a complete medium MEMD supplemented with 20% foetal calf serum for 0.5–2.0 h at 22°C min. and cryopreserved embryos (vitrified according to procedures described above) were used for transfers. Pregnancy of recipients was checked using ultrasonic probe in the 2nd month after ET.

Statistical analysis of data

All data were subjected to statistical analysis using a chi-square test or Student's *t*-test (Unistat, version 6.0).

RESULTS

In vitro culture of cryopreserved embryos

A majority portion of vitrified embryos was observed to develop during 36 hours *in vitro* culture. The percentage of developing embryos was 85% (17/20) for VS I protocol, 80% (16/20) for VS II protocol. Control embryos, which were not subjected to vitrification, developed in 95% (19/20) (Table 1).

Embryo transfer

One hundred and two embryos vitrified using VS I protocol were transferred into 7 recipients (14.6 ± 3.99 embryos per recipient on average). Ninety-three embryos vitrified using VS II protocol were transferred into 6 recipients (15.5 ± 4.28 embryos per recipient on average). Three hundred and twelve fresh embryos were transferred as the control group into 23 recipients (13.57 ± 5.83 embryos per recipient on average). Pregnancy was diagnosed in 5 recipients (57.3%) from VS I group, in 4 recipients (66.7%) from VS II group, and in 10 recipients (42.7%) from the control group. Differences between the groups were statistically significant ($P > 0.01$, Table 2).

Natality after transfer

Forty piglets were born after the transfer of embryos vitrified using VS I protocol (i.e. 51.6% of the transferred embryos). This represented 8.00 ± 2.00 piglets per sow. Thirty-seven piglets (92.50% of all piglets born) were born alive. Thirty-four piglets (91.9% of those born alive) were weaned (Table 2).

Thirty-one piglets were born after the transfer of embryos vitrified using VS II protocol (i.e. 46.3% of the transferred embryos). This represented 7.80 ± 1.26 piglets per sow. Twenty-nine piglets were born alive (93.5% of all piglets born). Twenty-six piglets (89.7% of those born alive) were weaned.

Sixty-five piglets were born after the transfer of fresh embryos (i.e. 37.4% of the transferred embryos). Sixty-two piglets (95.4%) were born alive, and all these piglets were weaned.

Some differences between experimental groups were statistically significant (Table 2).

DISCUSSION

In the present study we observed a very high ratio of *in vitro* development in embryos vitrified using our two vitrification protocols. These results are in agreement with the result of Misumi *et al*. (2003), who described development of pig embryos vitrified by the microdroplet method.

We also observed a very high pregnancy rate after the transfer of embryos vitrified using our two vitri-

Table 1. Development of vitrified and fresh porcine embryos after *in vitro* culture

Parameter	Type of embryos		
	VS I	VS II	Fresh (control)
Number of cultured embryos	20	20	20
Number of viable embryos after cultivation	17	16	19
Viability (%)	85	80	95

Differences between groups are statistically insignificant $P > 0.05$

Table 2. General results of the transfer of porcine fresh and vitrified embryos

Parameter		Type of embryos		
		VS I	VS II	Fresh (control)
Number of recipients (ET)	*n*	7	6	23
Pregnant recipients	*n*	5	4	10
Pregnancy rate	(%)	57.3[a]	66.7[f]	42.3[b,e]
Number of transferred embryos				
– total	*n*	102	93	312
	$\bar{x}$ ± sd	14.6 ± 3.99[b]	15.5 ± 4.28[a]	13.6 ± 5.38[b]
– from pregnant recipient	*n*	77	67	165
	$\bar{x}$ ± sd	15.4 ± 3.85	16.7 ± 4.27	16.5 ± 7.44
Natality-born piglets				
– total	*n*	40	31	65
	$\bar{x}$ ± sd	8.0 ± 2.0[a]	7.8 ± 1.26[a]	6.5 ± 2.42[b]
– alive	*n*	37	29	62
	$\bar{x}$ ± sd	7.4 ± 2.41[c]	7.3 ± 1.5[c]	6.2 ± 2.39 [d]
– weaned	*n*	34	26	62
	$\bar{x}$ ± sd	6.8 ± 1.64	6.5 ± 0.58	6.2 ± 2.39
Rate				
– born piglets from total transferred embryos from pregnant recipients	*n*	40/77	31/67	65/165
	(%)	51.6	46.3	37.4
– born alive piglets from embryos transferred to pregnant recipients	*n*	37/77	29/67	62/165
	(%)	48.1	43.3	37.6
– weaned piglets from embryos transferred to pregnant recipients	*n*	34/77	26/67	62/165
	(%)	44.2	38.8	37.6
– piglets born alive from total born	*n*	37/40	29/31	62/65
	(%)	92.5	93.5	95.4
– weaned piglets from born alive	*n*	34/37	26/29	62/62
	(%)	91.9[e]	89.7[e]	100[f]

[ab]$P < 0.01$; [cd]$P < 0.05$; [ef]$P < 0.001$

fication protocols. Pregnancy rates reported in this study were better than or comparable to data reported by Reichenbach *et al*. (1993), Galvin *et al*. (1994), Modl (1994), Hazeleger *et al.* (1995a,b), Wallenhorst and Holtz (1995), Gordon (1997), Cuello *et al*. (2002), Fujino *et al*. (2003). Similar results were also found in our previous studies (Říha *et al*., 1996, 1997a,b; Říha and Vejnar, 2000, 2003).

In our experiments, we did not investigate the effect of the parity of recipients (sows vs. gilts). This approach is based on our previous study (Říha and Vejnar, 2003), which clearly demonstrated that there was no difference in pregnancy rate, embryo survival, natality or rate of weaned piglets between recipient gilts and recipient sows regardless of the type of transferred embryos (cryopreserved vs. fresh ones). It is generally concluded that both gilts and sows can be used as recipients for embryos transfer with satisfactory results. The key factor in success is the precise control of oestrus (onset and intensity) fol-

lowing piglet weaning or synchronization treatment (Gordon, 1997). Based on these recommendations, we preferred the recipients with very good signs of oestrus for the transfer of vitrified embryos, and we also transferred significantly higher numbers of vitrified embryos when compared to the transfer of fresh embryos.

Our results are surprising with regard to widely reported problems accompanying the cryopreservation of porcine embryos. Based on these results, we support the ideas outlined by Caamano *et al.* (2003), who suggested the continuation of experimental work focused on commercialization of porcine ET. On the other hand, we would like to stress that so far the studies on ET in pigs have not gone beyond the stage of basic research. The same situation is met in cryopreservation of porcine embryos, which is also accompanied by numerous problems (Niemann, 1995; Gordon, 1997, our previous studies). Our previous paper (Říha *et al.*, 1997a) mentioned the birth of the first piglets produced after ET of cryopreserved embryos. However, other data published on laparoscopic ET in swine are limited. Thirteen laparoscopic transfers of vitrified embryos (the method specified by Říha *et al*., 1996, 1997a,b) to the recipients were reported in our earlier paper (Říha and Vejnar, 2000). However, our present results are substantially better than those achieved in our previous studies (Říha *et al*., 1997b; Říha and Vejnar, 2000) performed on much smaller sets of recipients. Natality and weaning rates in our study are comparable to the rates found after the surgical transfer of fresh pig embryos (Gordon, 1997). With respect to these facts, we can classify the results of our study as very successful.

Polge and Day (1968) published the first results (very low, approx. 3%) of non-surgical transfers. Sims and First (1987) used a non-surgical method for ET in pigs without any success. Reichenbach *et al*. (1993), Galvin *et al*. (1994), Modl (1994), Hazeleger *et al*. (1995a,b), Wallenhorst and Holz (1995) successfully used non-surgical methods for transcervical transfers of fresh porcine embryos using a catheter which was similar to the catheter used in our experiments. Li *et al.* (1995) and Gordon (1997) mentioned the importance of catheter construction for the success of ET in pigs. Cameron and van der Lende (1998) presented an even more successful non-surgical procedure ET program, although their experiments were not done on frozen porcine embryos but on fresh ones. Yonemura *et al*. (2003) found the highest pregnancy rate after non-surgical transfer utilizing a conventional catheter and 15 ml transfer medium (to standing anaesthetised recipients). Cuello *et al*. (2002) transferred vitrified embryos with a catheter used for deep intrauterine insemination. This method was successful – five of eight recipients were pregnant.

The complex method for porcine ET is still under development, but it is not losing appeal because it promises several advantages, e.g. it does not need pharmacological sedation of recipients. Based on our experience and our unpublished results we can state that the use of intrauterine insemination catheter for non-surgical ET is very promising.

Based on the data from our study, we can conclude that both tested protocols for the vitrification yielded similar results and can be used for cryopreservation of porcine embryos with satisfactory results after ET of thawed embryos.

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ABSTRAKT

Porovnání úspěšnosti dvou postupů vitrifikace při kryokonzervaci embryí prasat

Cílem práce bylo porovnání úspěšnosti dvou postupů vitrifikace embryí prasat. Embrya ve stadiu expandované blastocysty a kolem opuštění zony pellucidy byla získávána *post mortem* od superovulovaných dárkyň – vyřazených plemenných prasniček plemene landrace a české výrazně masné 5,5 až 6 dní po říji a inseminaci homospermními inseminačními dávkami. Ošetření dárkyň zahrnovalo zkrmování přípravku Regumate (Hoechst) podle návodu výrobce a ošetření PMSG (Sergon, Bioveta Ivanovice na Hané) a HCG (Praedyn, Léčiva Praha nebo Werfachor, Austria). Recipientky – prasničky byly synchronizovány zkrmováním přípravku Regumate a ošetřeny preparáty

PMSG a HCG; recipientky – prasnice po 1. a 2. vrhu byly synchronizovány odstavem selat mezi 28. a 32. dnem po porodu. Pouze recipientky, které vykázaly říji a reflex nehybnosti v čase inseminace dárkyň, byly použity k ET. Embrya odpovídajících vývojových stadií byla konzervována ve dvou vitrifikačních mediích: VS I – vitrifikační médium složené z 30 % glycerolu (G), 20 % fetálního telecího séra (FCS) a 50 % 2M sacharózy v tridestilované vodě (max. 1,5 min); k ekvilibraci embryí bylo použito médium H-MEMD s 10 % glycerolu po dobu 10 minut. VS II – vitrifikační médium obsahovalo 25 % dimethylsulfoxidu (DMSO), 25 % ethylenglykolu (EG) v mediu H-MEMD s 20 % FCS (max. 1,5 min). Ekvilibrace embryí probíhala po dobu 10 min ve směsi vitrifikačního a kultivačního média v poměru 1 : 1. Potom byla u obou postupů embrya nakapána mikropipetou do lázně tekutého dusíku a v něm uložena. Rozmrazování a odmývání embryí bylo prováděno vhozením kapky vitrifikačního média s embryi do kapky média H-MEMD s 0,8M obsahem sacharózy na Petriho misce po dobu 8 až 10 minut. Potom byla třikrát promyta v čerstvém kultivačním médiu a připravena pro kultivaci v podmínkách *in vitro* (uzavřené pejety o obsahu 0,5 ml, kultivační kondiciované médium H-MEMD s 20 % FCS, 24 až 36 hodin) nebo pro přenos příjemkyním. Pro nechirugické přenosy byl použit katetr pro intracervikální inseminaci, kterým byla provlečna silikonová, přiměřeně rigidní hadička naplněná embryi a napojená na injekční stříkačku s cca 5 ml média pro naplavení embryí do děložních rohů. Podíl vyvíjejících se nebo reexpandovaných rozmrazených embryí po kultivaci v *in vitro* podmínkách činil 85 % (VS I) a 80 % VS II), kontrolních čerstvých embryí 95 % ($P > 0,05$). Zabřezávání příjemkyň činilo v pořadí skupin 57,3 % (5/7), 66,7 % (4/6) a 42,7 % (10/23, $P < 0,05$). Poněkud více vitrifikovaných než čerstvých embryí bylo přenášeno u všech použitých i zabřezlých recipientek. Rozdíly v podílech selat narozených celkem, živě a odstavených z přenesených embryí byly většinou statisticky neprůkazné. Lepší výsledky zabřezávání příjemkyň a přežívání embryí pokusných skupin byly dosaženy patrně proto, že experimentální embrya byla přenášena ve větším počtu.

Klíčová slova: prase; embryo; transfer; kryokonzervace; vitrifikace; příjemkyně; prasnička; prasnice; natalita

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