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Cryopreservation of equine embryos

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Summary

Economic success of equine embryo transfer is limited by several unique aspects of reproductive physiology of mares such as variable response to protocols for oestrous cycle synchronization and superovulation. To become independent from oestrous cycle synchronization, frozen embryos can be stored in liquid nitrogen until a suitable recipient mare is available for transfer. In principle, two methods are available for cryopreservation of equine embryos. In conventional (slow) freezing the embryo is dehydrated by subsequent equilibration in increasing concentration of cryoprotective additives. Cooling rates provided by an automatic freezer avoid intracellular crystallisation, which causes damage of cell membranes and organelles. Another procedure is the rapid freezing or vitrification. Very high cooling rates result in a glass-like state without ice formation. Unfortunately, only protocols for the cryopreservation of small equine embryos (<300µm diameter) have been successfully established by now.

Keywords: horse, reproduction, embryo, cryopreservation, conventional freezing, vitrification

Kryokonservierung equiner Embryonen

Der Erfolg von Embryotransferprogrammen beim Pferd ist durch die reproduktionsphysiologischen Besonderheiten bei der Stute, wie unbefriedigende Reaktionen auf Zyklussynchronisations- und Superovulationsprogramme, begrenzt. Während gekühlte Embryonen nur wenige Tage überleben, eröffnet das Einfrieren von Embryonen die Möglichkeit, auf den geeigneten Zyklusstand der Empfängerstute zu warten und den Transfer unter optimalen Bedingungen durchzu-führen. Grundsätzlich gibt es zwei Möglichkeiten der Kryokonservierung. Beim konventionellen oder langsamen Tiefgefrierverfahren wird der Embryo zunächst durch das mehrmalige Umsetzen in Kryoprotektiva zunehmender Konzentration dehydriert. Anschließend wird er mit Hilfe von Einfriergeräten mit entsprechenden Temperaturkurven auf -30 bis -38°C abgekühlt und danach in flüssigem Stickstoff bei -196°C gelagert. Während des Einfriervorgangs muss die Bildung von großen intrazellulären Eiskristallen verhindert werden, da dies die Zellmembranen und Organellen zerstören würde. Die Alternative zu diesem Verfahren ist das Vitrifizieren der Embryonen. Dabei werden sehr hohe Kühlraten erreicht, wodurch es zu einem glasähnlichen Zustand ohne Bildung von Eiskristallen kommt. Vitrifizieren ist schneller und kostengünstiger, allerdings sind sehr hohe Konzentrationen der Kryoprotektiva erforderlich, die wiederum für den Embryo toxisch sein können. Unabhängig vom Verfahren war das Tiefgefrieren bisher nur bei kleinen Embryonen (<300µm im Durchmesser) erfolgreich.

Schlüsselwörter: Pferd, Reproduktion, Embryo, Kryokonservierung, konventionelles Einfrieren, Vitrifizierung

Introduction

Embryo transfer has the potential to increase the number of foals per mare, providing a useful tool for enhancing the maternal impact on breeding. Successful nonsurgical transfer of fresh embryos in horses has been reported first by *Oguri* and *Tsutsumi* (1974). By now, pregnangy rates up to 100% after transfer of fresh embryos have been obtained (*Wilsher* et al. 2009). Cooled embryos yielded in pregnancy after storage for 84 hours (*Pashen* 1987). Cooling equine embryos for 24 hours resulted in a pregnancy rate of 70% after surgical transfer. However, increased early embryonic loss after cooling was noticed (*Carnevale* et al. 1987).

The first pregnancy from a cryopreserved equine embryo was reported by *Griffin* et al. (1981) and the birth of the first foal obtained from the transfer of a frozen embryo by *Yamamoto* et al. (1982). Two years later, *Takeda* et al. (1984) produced two live foals by surgical transfer of four frozen-thawed equine embryos. However, until 2000 only about 50 pregnancies and fewer than 10 foals from cryopreserved conceptuses have been reported (*Leibo* 2000). Big advantages of freezing embryos are the possibility for almost infinite storage and preservation of important genetic lines. Further, the number of recipients can be kept small and embryos can be transported over long distances, independent from oestrous cycle stage of the recipient (*McKinnon* and *Squires* 2009).

Cryopreservation of embryos comprises the following steps:

1. exposure to cryoprotective additives (CPAs)
2. cooling to sub-zero temperatures and dehydration of cells
3. immersion in liquid nitrogen at -196°C
4. warming to physiological temperature
5. removal of CPAs.

Each of these steps has the potential to damage or destroy mammalian embryos (*Leibo* 2000). Optimum freezing- and thawing-rates minimize ice formation and damage of cellular structures (*McKinnon* and *Squires* 2009). Chilling and freezing injuries of embryos depend on species, developmental stage, origin (in vivo or in vitro) and how long a critical temperature continues (*Leibo* et al. 1996). In practice, there are two methods for freezing embryos: conventional (slow) freezing and vitrification.

Cryopreservation procedures

Conventional (slow) freezing

With conventional freezing methods, low cooling rates controlled by programmable automatic freezers are applied to embryos after equilibration with increasing concentrations of intracellular and/or extracellular cryoprotectants. During slow cooling at approximately 0.5°C per minute, dehydration of the embryo and replacement of intracellular water by the cryoprotectant should prevent the formation of ice crystals and subsequent damage of embryonic cellular structures. Although numerous protocols have been developed for freezing embryos, most of them are based on more or less similar steps: 1) washing the embryo in holding medium, 2) evaluation of embryo quality (morphology), 3) equilibration in the freezing medium and loading the in 0.25mL straws at room temperature, 4) cooling to -5 to -7 °C, 6) seeding (induction of crystallisation), 7) slow cooling (0.1 to 0.5 °C/min) in one or two steps to -30 to -40 °C and 8) plunging the straws into liquid nitrogen (Table 1a-d). Structural damage of the zona pellucida was observed more frequently in embryos cooled to -40°C compared to embryos cooled to -35°C before plunging into liquid nitrogen (*Czlonkowska* et al. 1985). Packaging in straws and plunging into liquid nitrogen at -33°C was described as optimal method by *Slade* et al. (1985). The success rates of different conventional freezing protocols for equine embryos are listed in Table 1a-d.

From the practical point of view, slow freezing protocols have the disadvantages to be time consuming – at least 1.5 hours have to be considered – and to require expensive equipment such as programmable freezer.

Vitrification

Major advantages of vitrification are that no expensive equipment is required and that it is a rapid method suitable to be performed under field conditions. Very high cooling and warming rates are achieved by this method. Vitrification of embryos uses higher concentrations of cryoprotectants compared with conventional freezing protocols. Hence, solutions become more viscous and solidify without the formation of ice crystals leading to a glass-like state (*Carnevale* 2006). However, concentrated solutions of cryoprotectants have the potential to damage embryos either directly by toxic effects or indirectly by osmotic shock. Usually, three vitrification solutions are required with various combinations of glycerol and ethylene glycol as cryoprotectants (Table 2a-c).

Exposure of embryos to cryoprotectants is very critical and has to be carried out stepwise at precise intervals. At least, exposure to the last vitrification solution has to be kept as short as possible and should not exceed one minute. Vitrified embryos can be loaded into 0.25mL straws similar to conventional freezing method. Loaded straws are placed into nitrogen vapour for one minute and then plunged into liquid nitrogen. Loading the embryo into a straw allows to transfer the embryo directly after thawing to recipient mares (*Carnevale* et al. 2004) providing a good method for field embryo transfer resulting in good pregnancy rates for morulae and blastocysts smaller than 300µm (*Eldridge-Panuska* et al. 2005).

Other methods, which have been developed, are the open-pulled-straw (OPS) and the cryoloop technique (*Oberstein* et al. 2000, *Oberstein* et al. 2001, *Moussa* et al. 2004) aiming to reduce the size of the fluid compartment surrounding the embryo for acceleration of the freezing process. By the OPS method it is possible to reach cooling and warming rates of about 20,000°C/min. This is approximately tenfold higher than vitrification by straws. OPS were easy and rapid to load because of the capillary effects and reduced the risk of zona and embryo fracture. Dilution of the cryoprotectant additives occurs immediately during the warming process. The only disadvantage of OPS is the direct contact of the embryo holding medium with liquid nitrogen, which should be sterile in that case (*Vajta* et al. 1998).

Small equine embryos can be stored at 5-8°C for 12 to 19 hours before vitrification without loss of viability. Therefore, the transport of the embryos to the laboratory for vitrification is possible (*Hudson* et al. 2006).

Vitrification of large embryos has not been successful (*Carnevale* et al. 2004, *Eldridge-Panuska* et al. 2005). Large equine embryos had the lowest viability rate in vitro and the highest fracture rate after thawing (*Hochi* et al. 1995).

Thawing of frozen embryos

Thawing of cryopreserved embryos is mostly performed in a two-step procedure, starting with a short exposure to air at room temperature for 5 to 12 seconds followed by thawing in a water-bath at temperatures from 20 to 37°C for 10 to 60 seconds (Tables 1 and 2). Regardless of the freezing method, the cryoprotectant has to be removed from the embryo after thawing. Two different strategies have been developed: the stepwise dilution by transferring the embryo step-by-step to media each containing lower concentrations of the cryoprotectant or the direct transfer method. For the latter, the 0.25mL polyvinyl straw is loaded with only a small column of freezing medium and a major portion of holding medium separated by small air bubbles. After thawing, the straw has to be flicked to mix the compartments for dilution of the embryo before transfer. This method provides by means of the use at field conditions significant advantage compared with stepwise dilution, because there is no need for additional equipment. Further, the cessation of stepwise dilution decreases the risk of contamination and saves time.

Discussion

Success of cryopreservation depends on size and developmental stage of the embryos. Only conceptuses smaller than 300µm in diameter yielded acceptable survival and/or pregnancy rates (*Slade* et al. 1985, *Campos-Chillon* et al. 2006, *Carnevale* 2006). Morula and early blastocyst stage were associated with better embryo quality after freezing and thawing and provided a better potential for the embryos to survive after in vitro culture (*Poitras* et al. 1994). Small embryos resulted in pregnancy rates of around 70% after transfer compared with pregnancy rates between 10 and 20% in larger expanded blastocysts exceeding 300µm in diameter (*McKinnon* and *Squires* 2009).

Table 1a Conventional freezing procedures and survival rate of embryos

Authors	n	ER	size (µm)	cryoprotectant	cooling protocol °C (cooling rate °C/min)	thawing/temperature °C (rate °C)/CPA removal	SR (%)
Takeda et al. 1984	7	D6	n.i.	1.32M gly in 3 equimolar steps (*10-10-30 min*)	n.i.; -7 **S** (-0.3); -26 (-0.1);-38 **N**	**37**;	57.1
	7	D6	n.i.	1.32M gly in 3 equimolar steps (*10-10-30 min*)	n.i.;-7 **S** (-0.3); -35 (-0.1);-38 **N**	**37**;	71.4
	7	D7	n.i.	1.32M gly in 3 equimolar steps (*10-10-30 min*)	n.i.; -7 **S** (-0.3); -26 (-0.1);-38 **N**	**37**;	14.3
	7	D7	n.i.	1.32M gly in 3 equimolar steps (*10-10-30 min*)	n.i.; -7 **S** (-0.3); -35 (-0.1);-38 **N**	**37**;	28.6
Slade et al. 1985	8	D6	<260	*10min* 5% gly - *20-30min* 10% gly [9]	**RT** (-0.4); -6.0 **S** (-0.3); -30 (-0.1); -33 **N**	wb **37**; 10.0-8.3-6.7-5.0-3.3-1.6-0% gly each *10min*	88.0
	8	D6	<260	*10min* 5% gly - *20-30min* 10% gly [9]	**RT** (-0.4); -6.0 **S** (-0.3); -35 (-0.1); -38 **N**	wb **37**; 10.0-8.3-6.7-5.0-3.3-1.6-0% gly each *10min*	62.0
	8	D6	<260	*10min* 5% gly - *20-30min* 10% gly [3]	**RT** (-0.4); -6.0 **S** (-0.3); -30 (-0.1); -33 **N**	wb **37**; 10.0-8.3-6.7-5.0-3.3-1.6-0% gly each *10min*	50.0
	8	D6	<260	*10min* 5% gly - *20-30min* 10% gly [3]	**RT** (-0.4); -6.0 **S** (-0.3); -35 (-0.1); -38 **N**	wb **37**; 10.0-8.3-6.7-5.0-3.3-1.6-0% gly each *10min*	12.0
Poitras et al. 1994	25	D6.5	<220	*10min* 0.7M (5%) gly 1.4M - *20min* (10%) gly	**25±2** (-4.0); -7.0 **S** -7.0 (*15min*) (-0.3); -30 (*30min*) **N**	*10s* wb **37**; 1.4M gly+4g/l BSA **37**; 1.165-0.938-0.7-0.462-0.224-0.0M gly each *10min*	27.7
	20	D6.5	<220	*10min* 0.7M (5%) gly 1.4M - *20min* (10%) gly [4]	**25±2** (-4.0); -7.0 **S** -7.0 (*15min*) (-0.3); -30 (*30min*) **N**	*10s* wb **37**; 1.4M gly+4g/l BSA **37**; 1.165-0.938-0.7-0.462-0.224-0.0M gly each *10min*	22.5
	22	D6.5	<220	*10min* 0.7M (5%) gly 1.4M - *20min* (10%) gly	**25±2** (-4.0); -7.0 **S** -7.0 (*15min*) (-0.3); -30 (*30min*) **N**	*10s* wb **37**; 1.4M gly+4g/l BSA **37**; 1.165-0.938-0.7-0.462-0.224-0.0M gly each *10min*	27.7
	20	D6.5	<220	*10min* 0.7M (5%) gly 1.4M - *20min* (10%) gly [4]	**25±2** (-4.0); -7.0 **S** -7.0 (*15min*) (-0.3); -30 (*30min*) **N**	*10s* wb **37**; 1.4M gly+4g/l BSA **37**; 1.165-0.938-0.7-0.462-0.224-0.0M gly each *10min*	22.5
	13	D6.5	>220	*10min* 0.7M (5%) gly 1.4M - *20min* (10%) gly	**25±2** (-4.0); -7.0 **S** -7.0 (*15min*) (-0.3); -30 (*30min*) **N**	*10s* wb **37**; 1.4M gly+4g/l BSA **37**; 1.165-0.938-0.7-0.462-0.224-0.0M gly each *10min*	0.0
	7	D6.5	>220	*10min* 0.7M (5%) gly 1.4M - *20min* (10%) gly [4]	**25±2** (-4.0); -7.0 **S** -7.0 (*15min*) (-0.3); -30 (*30min*) **N**	*10s* wb **37**; 1.4M gly+4g/l BSA **37**; 1.165-0.938-0.7-0.462-0.224-0.0M gly each *10min*	0.0
	18	D6.5	>220	*10min* 0.7M (5%) gly 1.4M - *20min* (10%) gly	**25±2** (-4.0); -7.0 **S** -7.0 (*15min*) (-0.3); -30 (*30min*) **N**	*10s* wb **37**; 1.4M gly+4g/l BSA **37**; 1.165-0.938-0.7-0.462-0.224-0.0M gly each *10min*	0.0
	4	D6.5	>220	*10min* 0.7M (5%) gly 1.4M - *20min* (10%) gly [4]	**25±2** (-4.0); -7.0 **S** -7.0 (*15min*) (-0.3); -30 (*30min*) **N**	*10s* wb **37**; 1.4M gly+4g/l BSA **37**; 1.165-0.938-0.7-0.462-0.224-0.0M gly each *10min*	0.0
Young et al. 1997	7	D7-8	471±45	*15min* 1.0M gly	**RT** (-4.0); -6.0 (*5min*) **S** -6.0 (*5min*) (-0.5); -35 (*3min*) **N**	*12s* **air** - *12s* wb **37**; 0.6-0.4-0.2-0.0M gly each *5min*; **20-21**	57.1
	6	D7-8	518±39	2.0M gly - 4.0M gly - 2.0M gly+0.3M gal each *5min*	**RT** (-4.0); -6.0 (*5min*) **S** -6.0 (*5min*) (-0.5); -35 (*3min*) **N**	*12s* **air** - *12s* wb **37**; 1.5+0.3-0.8 +0.3-0.3+0.3-0.0+0.3 (M gly+M gal) each *5min*; **20-21**	85.7
Lagneaux et al. 1997	3	D6	160-190	2.5-5.0-7.5-10.0 % gly each *20min*	**RT** (-3.0); -7.0 **S** (-0.3); -30 **N**	*30s* wb **37**; 8.3-6.6-5.0-3.3-1.6-0.0% gly each *30min*	0.0
	3	D6	150-170	*20min* 20mg/ml AFP	**RT** (-3.0); -7.0 **S** (-0.3); -30 **N**	*30s* wb **37**; PBS+1.3g/l PVP *5min*	0.0
	3	D6	180-200	2.5-5.0-7.5% gly each *15min* - *5min* 10.0% gly+20mg/ml AFP	**RT** (-3.0); -7.0 **S** (-0.3); -30 **N**	*30s* wb **37**; 8.3-6.6-5.0-3.3-1.6-0.0% gly each *30min*	0.0
O'Donovan et al. 2000	8	D7	300-600	*10min* 0.3M gal - *40min* 2.0M EG+0.3M gal	n.i. (-4.0); -6.0 (*5min*) **S** (-0.5); -35 **N**	*15s* **air** - *15s* wb **37**; CPAs removed in 3 or 4 steps	50.0
	5	D7	300-600	*10min* 0.3M gal - *15min* 2.0M EG+0.3M gal - *25min* 4.5M EG+0.3M gal	n.i. (-4.0); -6.0 (*5min*) **S** (-0.5); -35 **N**	*15s* **air** - *15s* wb **37**; CPAs removed in 3 or 4 steps	20.0
	8	D7	300-600	*10min* 0.3M gal - *10min* 2.0M EG+0.3M gal - *15min* 4.5M EG+0.3M gal – *10min* 2.0M EG+0.8M gal	n.i. (-4.0); -6.0 (*5min*) **S** (-0.5); -35 **N**	*15s* **air** - *15s* wb **37**; CPAs removed in 3 or 4 steps	0.0
Moussa et al. 2004	18	D6.5/6.75	183±7	2.5-5.0-7.5-10% gly each *5min*	**22-25** (-3.0); -7.0 **S** (-0.3); -30 N	*30s* wb **37**; 7.5+0.25-5.0+0.1-2.5+0.1-0.0+0.1 (% gly+mM suc)- each *5min*; **RT**	33,3

Table 1b Conventional freezing procedures and proportion of pycnotic cells after thawing

Authors	n	ER	size (µm)	cryoprotectant	cooling protocol °C (cooling rate °C/min)	thawing/temperature °C (rate °C)/CPA removal	pycnotic cells (%)
Bruyas et al. 1993	5	D6.5	198.9±36.5	2.5-5.0-7.5-10.0% gly each *5min*	**22** (-3.0); -7.0 **S** (*5min*) (-0.3); -30 **N**	*60s* wb **37**; 10.0-8.3-6.7-5.0-3.3-1.6-0.0% gly each *5min*	42.2±14.9
Bruyas et al. 1997a	5	D6	152-267	0.5-1.0-1.5M PD each *10min*	**22** (-3.0); -7.0 **S** (*5min*) (-0.3); -30 **N**	*60s* wb **37**; *10min* 1.5 M PD **22** - 1.0-0.5-0.0 M PD each *10min*	52.8±37.1
Bruyas et al. 1997b	6	D6.5	n.i.	0.5-1.0-1.5 M DMSO each *10min*	**22**; -7.0 **S** (-0.3); -35 **N**	*1min* wb **37**; 1.5-1.0-0.5-0.0 M DMSO	82.2±16.2
Bruyas et al. 2000	3	D6	161.5-184	0.375-0.75-1.125-1.5 M gly each *10min*	**22** (-3.0); -7.0 **S** -7.0 (*5min*) (-0.3); -30 **N**	*60s* wb **37**; 1.5-1.125-0.75-0.375-0.0 M gly each *10min*; **22**	1.1±0.8
	5	D6	165.6-211.1	0.375-0.75-1.125-1.5 M gly each *10min*	**22** (-3.0); -7.0 **S** -7.0 (*5min*) (-0.3); -30 **N**	*60s* wb **37**; 1.25+0.5-0.83+0.25-0.42+0.25-0.0+0.25-0.0+0.0 (M gly + M suc) each *10min*; **22**	2.5±1.5
	4	D6	170-185	0.5-1.0-1.5 M EG each *10min*	**22** (-3.0); -7.0 **S** -7.0 (*5min*) (-0.3); -30 **N**	*60s* wb **37**; 1.5-1.0-0.5-0.0 M EG each *10min*; **22**	n.n.
	5	D6	151.5-204	0.5-1.0-1.5 M EG each *10min*	**22** (-3.0); -7.0 **S** -7.0 (*5min*) (-0.3); -30 **N**	*60s* wb **37**; 1.25+0.25-0.6+0.25-0.0+0.25-0.0+0.0 (M EG + M suc) each *10min*; **22**	n.n.

Table 1c Conventional freezing procedures and proportion of viable cells after thawing

Authors	n	ER	size (µm)	cryoprotectant	cooling protocol °C (cooling rate °C/min)	thawing/temperature °C (rate °C)/CPA removal	viable cells (%)
Oberstein et al. 2000	8	D6-7	257	*10min* 1.8M EG+0.1M suc	**30; 0** (-0.5); -6.0 **S** -6.0 (*1min*) (-0.3); -35 **N**	*10s* **air** - *20s* wb **37;** H-SOF *3x5min*	74
Oberstein et al. 2001	8	D6-7	<300	*10min* 1.8M EG+0.1M suc	**30; 0** (-0.5); -6.0 **S** -6.0 (*1min*) (-0.3); -35 **N**	*10s* **air** - *20s* wb **37**; H-SOF *3x5min*	74

Table 1d Conventional freezing procedures and morphological score

Authors	n	ER	size (µm)	cryoprotectant	cooling protocol °C (cooling rate °C/min)	thawing/temperature °C (rate °C)/CPA removal	score (mean)
Seidel et al. 1989	8	D6.5-7	250-890	*5min* 5% PD - *10-20min* 10% PD	**20-22** (-4.0); -6.0 (*5min*) **S** -6.0 (*10min*) (-0.8); -33 **N**	*20s* wb **37**; 6.0+10.0-3.0+10.0-0.0+10.0 (% PD+% suc) each *6min*; **RT**	3.5
	5	D6.5-7	275-575	*5min* 5% PD - *10-20min* 10% PD	**20-22** (-4.0); -6.0 (*5min*) **S** -6.0 (*10min*) (-0.3); -33 **N**	*20s* wb **37**; 6.0+10.0-3.0+10.0-0.0+10.0 (% PD+% suc) each *6min*; **RT**	4.1
	5	D6.5-7	220-700	*5min* 5% PD - *10-20min* 10% PD	**20-22** (-4.0); -6.0 (*5min*) **S** -6.0 (*10min*) (-0.8); -33 **N**	**air**; 6.0+10.0-3.0+10.0-0.0+10.0 (% PD+% suc) each *6min*; **RT**	3.7
	5	D6.5-7	300-945	*5min* 5% PD - *10-20min* 10% PD	**20-22** (-4.0); -6.0 (*5min*) **S** -6.0 (*10min*) (-0.3); -33 **N**	**air**; 6.0+10.0-3.0+10.0-0.0+10.0 (% PD+% suc) each *6min*; **RT**	3.6

Table 1e Conventional freezing procedures and pregnancy rate

Authors	n	ER	size (µm)	cryoprotectant	cooling protocol °C (cooling rate °C/min)	thawing/temperature °C (rate °C)/CPA removal	PR (%)
Yamamoto et al. 1982	12	D6	n.i.	*10min* 0.5M DMSO - *10min* 1.0M DMSO - *30min* 1.5M DMSO [1,7]	**20** (-0.5 to -1.0); -5.0 **S** (*10min*) (-0.17 to -0.22); -70 **N**	**N** (+95) -70; (1.4-10.0) -10.0; (+8.0) **20;** 1.25-1.0-0.75-0.5-0.25-0.0M DMSO each *10min*	0.0
	11	D6	n.i.	*10min* 0.5M gly - *30min* 1.0M gly [1,7]	**20** (-0.5 to -1.0); -5.0 **S** (*10min*) (-0.17 to -0.22); -70 **N**	**N** (+95) -70; (1.4-10.0) -10.0; (+8.0) **20;** 0.5-0.0M gly each *10min*	27.3 (D32-36); 9.0 [f]
	4	D8	n.i.	*10min* 0.5M DMSO - *10min* 1.0M DMSO - *30min* 1.5M DMSO [2,7]	**20** (-0.5 to -1.0); -5.0 **S** (*10min*) (-0.17 to -0.22); -70 **N**	**N** (+95) -70; (1.4-10.0) -10.0; (+8.0) **20;** 1.25-1.0-0.75-0.5-0.25-0.0M DMSO each *10min*	0.0
	5	D8	n.i.	*10min* 0.5M gly - *30min* 1.0M gly [2,7]	**20** (-0.5 to -1.0); -5.0 **S** (*10min*) (-0.17 to -0.22); -70 **N**	**N** (+95) -70; (1.4-10.0) -10.0; (+8.0) **20;** 0.5-0.0M gly each *10min*	0.0
Takeda et al. 1984	2	D6	n.i.	1.32M gly in 3 equimolar steps (*10-10-30min*)	n.i.; -7 **S** (-0.3); -26 (-0.1);-38 **N**	**37**;	50.0 [f]
	2	D6	n.i.	1.32M gly in 3 equimolar steps (*10-10-30min*)	n.i.; -7 **S** (-0.3); -35 (-0.1);-38 **N**	**37**;	50.0 [f]
Czlonkowska et al. 1985	7	D6.5-7	uBL-exBl	2.5-5.0-7.5-10% gly each *10min*	**24** (-1.0); -6.0 **S** (0.0 to -3.0); -35 **N**	wb **37**; 10.0-7.5-5.0-2.5-0.0 % gly each *10min*	14.3 [f]; 28.6 (D66-74)
	7	D6-8	Mo-exBl	2.5-5.0-7.5-10% gly each *10min*	**24** (-1.0); -6.0 **S** (0.0 to -3.0); -40 **N**	wb **37**; 10.0-7.5-5.0-2.5-0.0 % gly each *10min*	0.0 [f]; 28.6 (D28-40)
Slade et al. 1985	17	D6	<220	*10min* 5% gly - *20-30min* 10% gly [9]	**RT** (-0.4); -6.0 **S** (-0.3); -30 (-0.1); -33 **N**	wb **37**; 10.0-8.3-6.7-5.0-3.3-1.6-0% gly each *10min*; **RT**	39.1 (D50)
Hochi et al. 1996	8	D6	Bl	5% EG - 10% EG each *10min*	**30; 0** (-0.5); -6.0 **S** (-0.3); -35 **N**	wb **37**; 8.3-6.7-5.0-3.3-1.6-0% gly each *10min* **30** - 3x DMEM/10%FBS	25.0 (D60)
	8	D6	Bl	5% gly - 10% gly each *10min*	**30; 0** (-0.5); -6.0 **S** (-0.3); -35 **N**	wb **37**; 8.3-6.7-5.0-3.3-1.6-0% gly each *10min* **30** - 3x DMEM/10%FBS	37.5 (D60)
	11	D6	Bl	*10min* 10% EG + 0.1M suc	**30; 0** (-0.5); -6.0 **S** (-0.3); -35 **N**	wb **37**; mixed with DMEM/10%FBS additionally loaded in the straw	36.4 (D60)
Ferreira et al. 1997	15	D6	Mo, eBl	*10min* 0.75M gly - *20min* 1.5M gly+0.25M suc	**RT** (-3.0); -6.0 (*5min*) **S** (*10min*) (-0.4); -30 **N**	*20s* wb **37**; 0.75+0.25-0.0+0.25-0.0+0.0 (M gly+M suc) each *10min*	13.3 (D15)
	7	D6	Mo, eBl	*10min* 0.35M gly + 0.35M PD - *20min* 0.75M gly+ 0.75M PD	**RT** (-3.0); -6.0 (*5min*) **S** (*10min*) (-0.4); -30 **N**	*20s* wb **37**; 0.5+0.5-0.375+0.375-0.25+0.25-0.125+0.125-0.0+0.0 (M gly+ M PD) each *10min*	0.0 (D15)
Huhtinen et al. 1997	20	D6	130-200	2.5-5.0-7.5-10.0% gly each *5min*	**RT** (-3.0); -7.0 **S** (*5min*) (-0.3); -30 **N**	*30s* wb **37**; 8.3-6.6-5.0-3.3-1.6-0.0% gly each *5min*; **RT**	30.0 (D20)
	20	D6	130-200	2.5-5.0-7.5-10.0% gly each *5min*	**RT** (-3.0); -7.0 **S** (*5min*) (-0.3); -30 **N**	*30s* wb **37**; 10.0+0.25-7.5+0.25-5.0+0.1-2.5+0.1-0.0+0.1 (% gly+M suc) each *5min*; **RT**	15.0 (D20)
Young et al. 1997	6	D7-8	300-680	2.0M gly - 4.0M gly - 2.0M gly+0.3M gal each *5min*	**RT** (-4.0); -6.0 (*5min*) **S** -6.0 (*5min*) (-0.5); -35 (*3min*) **N**	*12s* **air** - *12s* wb **37**; 1.5+0.3-0.8+0.3-0.3+0.3-0.0+0.3 (M gly+M gal) each *5min*; **20-21**	33.3 (D14)
Huhtinen et al. 2000	9	n.i.	140-190	gly+50mM glutamine in 4 steps	n.i.	*10s* **air** - *30s* wb; 10.0+0.25-7.5+0.25-5.0+0.1-2.5+0.1-0.0+0.1 (% gly+M suc) each *5min*; **RT**	44.4
	10	n.i.	140-190	1.5M EG in Emcare	n.i.	*10s* **air** - *30s* wb **30**	33.3
Legrand et al. 2000	8	D5.5-8.5	187-1581	0.5-1.0-1.5M gly each *15min* [5]	n.i.; -7.0 S (-0.3); N	*1min* wb **37**; 1.0+0.25-0.5+0.25-0.0+0.25 (M gly+M suc) each *15min*	75.0 (D14)
Legrand et al. 2002	8	D7-8	>200	0.5-1.0-1.5M gly each *15min*	n.i; -7.0 **S** (-0.3); -30 **N**	*1min* wb **37**; 1.0+0.25-0.5+0.25-0.0+0.25 (M gly+M suc) each *15min*	0.0
	11	D7-8	>200	0.5-1.0-1.5M gly each *15min* [5]	n.i.; -7.0 **S** (-0.3); -30 **N**	*1min* wb **37**; 1.0+0.25-0.5+0.25-0.0+0.25 (M gly+M suc) each *15min*	27.0 (D21-28)
Maclellan et al. 2002	9	D7.5-8.5	<300	*10min* 5% gly - *20min* 10% gly	**22** (-4.0); -6.0 **S** -6.0 (*10min*) (-0.3); -30 (-0.1); -33 **N**	*10s* **air** - *30s* wb **37**; 8.0-6.0-4.0-2.0-1.0-0.0% gly each *10min*; DT	55.5 (D16)
	8	D7.5-8.5	<300	*10min* 5% gly - *20min* 10% gly	**22** (-4.0); -6.0 **S** -6.0 (*10min*) (-0.3); -30 (-0.1); -33 **N**	*10s* **air** - *30s* wb **37**; 8.0-6.0-4.0-2.0-1.0-0.0% gly each *10min*; culture *6h* -transfer	87.5 (D16)
	7	D7.5-8.5	>300	*10min* 5% gly - *20min* 10% gly	**22** (-4.0); -6.0 **S** -6.0 (*10min*) (-0.3); -30 (-0.1); -33 **N**	*10s* **air** - *30s* wb **37**; 8.0-6.0-4.0-2.0-1.0-0.0% gly each *10min*	57.1 (D16)
	7	D7.5-8.5	>300	*10min* 5% gly - *20min* 10% gly	**22** (-4.0); -6.0 **S** -6.0 (*10min*) (-0.3); -30 (-0.1); -33 **N**	*10s* **air** - *30s* wb **37**; 8.0-6.0-4.0-2.0-1.0-0.0% gly each *10min*	0.0 (D16)
	7	D7.5-8.5	>300	*10min* 5% gly - *20min* 10% gly [6]	**22** (-4.0); -6.0 **S** -6.0 (*10min*) (-0.3); -30 (-0.1); -33 **N**	*10s* **air** - *30s* wb **37**; 8.0-6.0-4.0-2.0-1.0-0.0% gly each *10min*	42.9 (D16)
	7	D7.5-8.5	>300	*10min* 5% gly - *20min* 10% gly [5,6]	**22** (-4.0); -6.0 **S** -6.0 (*10min*) (-0.3); -30 (-0.1); -33 **N**	*10s* **air** - *30s* wb **37**; 8.0-6.0-4.0-2.0-1.0-0.0% gly each *10min*	0.0 (D16)
Ulrich and *Nowshari* 2002	1	D7	eBl	*5-10min* 1.5M EG + 0.25M sac	n.i.;-4.5 (*10min*) **S** (*10min*) (-0.3); -35 **N**	*5-6s* **air** - *20s* wb **35**	100.0 (f)
Barfield et al. 2009	16	D7-8	400-1350	*2min* 0.6M gal - *10min* 1.5M gly+0.6M gal	**RT**; -6.0 **S** (-0.5); -32 **N**	*8s* **air** - *30s* wb **35**	6.25 (12.5)
	10	D7-8	400-950	*10min* 1.5M gly+0.6M gal	**RT**; -6.0 **S** (-0.5); -32 **N**	*8s* **air** - *30s* wb **35**	10 (20)
Meira et al. 1993	12	D6-7	Bl/exBl	*10min* 5%gly - *20-25min* 11% gly [9]	**22-27**; (-2.5 to -4.0) -6.0 **S** -6.0 to -7.0 (*10min*) (-0.3 to -0.6) -30; **N**	*20s* wb **37**; 7.3-5.5-3.7-1.8-0.0 % gly each *10min*	0.0
	15	D6-7	M/eBl	*10min* 5%gly - *20-25min* 11% gly [9]	**22-27**; (-2.5 to -4.0) -6.0 **S** -6.0 to -7.0 (*10min*) (-0.3 to -0.6) -30; **N**	*20s* wb **37**; 7.3-5.5-3.7-1.8-0.0 % gly each *10min*	40.0
	17	D6-7	M/eBl	*10min* 5%PD - *20-25min* 11% PD [9]	**22-27**; (-2.5 to -4.0) -6.0 **S** -6.0 to -7.0 (*10min*) (-0.3 to -0.6) -30; **N**	*20s* wb **37**; 7.3-5.5-3.7-1.8-0.0 % PD each *10min*	0.0

Table 2a Vitrification procedures and survival rate of embryos

Authors	n	ER	size (µm)	cryoprotectant	cooling protocol °C (cooling rate °C/min)	thawing/temperature (rate °C)/CPA removal	SR (%)
Hochi et al. 1994a	7	D5-7	169.3	*1-2min* 40% EG +18% ficoll+0.3M suc	0.25 straws; N-vapour *1min*; **N**	*20s* wb **20**; *10min* 0.5M suc - mPBS	0.0
	7	D5-7	169.3	*10-20min* 20% EG - *2min* 40% EG+18% ficoll+0.3M suc	0.25 straws; N-vapour *1min*; **N**	*20s* wb **20**; *10min* 0.5M suc - mPBS	57.1
	7	D5-7	169.3	*10-20min* 20% EG - *2min* 40% EG+18% ficoll+0.3M suc	0.25 straws; N-vapour *1min*; **N**	*20s* wb **20**; 0.5-0.38-0.25-0.13-0.0M suc each *10min*	57.1
Hochi et al. 1995	8	D6	156-199	*20min* 20% EG **25** - 40% EG+18% ficoll+0.3M suc	0.25 straws; N-vapour *1min*, **N**	*20s* wb **20**; DS 0.5M suc; *10min* 0.5M suc - mPBS	88.0
	8	D6-7	202-292	*20min* 20% EG **25** - 40% EG+18% ficoll+0.3M suc	0.25 straws; N-vapour *1min*, **N**	*20s* wb **20**; DS 0.5M suc; *10min* 0.5M suc - mPBS	75.0
	8	D7-8	313-980	*20min* 20% EG **25** - 40% EG+18% ficoll+0.3M suc	0.25 straws; N-vapour *1min*, **N**	*20s* wb **20**; DS 0.5M suc; *10min* 0.5M suc - mPBS	25.0
Young et al. 1997	7	D7-8	409±24	*15min* 4.5M EG - *1min* 11.9M EG+2.6mM ficoll+0.3M gal	0.25 straws; N-vapour *1min*, **N**	*12s* **air** - *12s* wb **37**; 0.6-0.3-0.15-0.0M gal each *15min*	71.4
Moussa et al. 2004	18	D6.5/6.75	187±6	*3min* 7.5% DMSO+7.5% EG - *1min* 18% DMSO+18% EG	OPS	*2s* **air**; *2x* TCM+0.13M suc - TCM + 0.075M suc - TCM each *5min*	45.0

Table 2b Vitrification procedures and proportion of viable cells after thawing

Authors	n	ER	size (µm)	cryoprotectant	cooling protocol °C (cooling rate °C/min)	thawing/temperature (rate °C)/CPA removal	viable cells (%)
Castanheira et al. 2004	6	D6/6.5	170-235	*10min* 20% EG - 40% EG	0.25 straws; N-vapour *1min*; **N**	*20s* **20** - *10min* 0.5M suc	0.8±1.1
	6	D6/6.5	170-235	*10min* 20% EG - 40% EG+18% ficoll+0.3M suc	0.25 straws; N-vapour *1min*; **N**	*20s* **20** - *10min* 0.5M suc	26.7±26.6
	6	D6/6.5	170-235	*10min* 20% EG - 40% EG+18% ficoll+0.3M trehalose	0.25 straws; N-vapour *1min*; **N**	*20s* **20** - *10min* 0.5M suc	19.2±8.01
Oberstein et al. 2000	11	D6-7	232	*3min* 7.5% DMSO+7.5% EG - *20-30s* 16.5% DMSO+16.5% EG+0.5M suc	OPS	*5min* 0.15M suc - H-SOF *2x5min*	51
	8	D6-7	211	*2.5min* 7.5% DMSO+7.5% EG - *20-30s* 17.5% DMSO+17.5% EG+1.0M suc+0.25µM ficoll	cryoloop	840µl H-SOF: *2.5min* +(420ml H-SOF+1.0M suc) - *3min* +(210ml H-SOF+1.0M suc) - *5min* H-SOF; **37**	48
Oberstein et al. 2001	11	D6-7	<300	*3min* 7.5% DMSO+7.5% EG - *20-30s* 16.5% DMSO+16.5% EG+0.5M suc	OPS	*1min* 0.25M suc **37 -** *5min* 0.15M suc - H-SOF *2x5min*	51
	8	D6-7	<300	*2.5min* 7.5% DMSO+7.5% EG - *20-30s* 17.5% DMSO+17.5% EG+1.0M suc+0.25µM ficoll	cryoloop	840µl H-SOF: *2.5min* +(420ml H-SOF+1.0M suc) - *3min* +(210ml H-SOF+1.0M suc) - *5min* H-SOF; **37**	48

Table 2c Vitrification procedures and pregnancy rate

Authors	n	ER	size (µm)	cryoprotectant	cooling protocol °C (cooling rate °C/min)	thawing/temperature (rate °C)/CPA removal	PR (%)
Hochi et al. 1994a	5	D6	169.3	*10-20min* 20% EG - *2min* 40% EG+18% ficoll+0.3M suc	0.25 straws; N-vapour *1min*; **N**	*20s* wb **20**; dilution in suc	40.0 (D60)
Carnevale et al. 2004	22	D6	186.1±8.6	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *<1min* 3.4M gly+4.6M EG	0.25 straws	*10s* **air** - *10s* wb **20**; *5min* 0.25M gal - *<10min* PBS	45.0 (D16)
	10	D6-8	575.0±82.0	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *<1min* 3.4M gly+4.6M EG	0.25 straws	*10s* **air** - *10s* wb **20**; *5min* 0.25M gal - *<10min* PBS	0.0 (D16)
	10	D6-8	643.5±83.3	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *<1min* 1.4M gly+6.6M EG	0.25 straws	*10s* **air** - *10s* wb **20**; *5min* 0.25M gal - *<10min* PBS	0.0 (D16)
	26	D6	181.7±5.4	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *<1min* 3.4M gly+4.6M EG	0.25 straws, add. 30µl 3.4M gly+4.6M EG	*10s* **air** - *10s* wb **20**; DS 0.5M gal; DT	62.0 (D16)
Eldridge-Panuska et al. 2005	6	D6.5-7.5	<300	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *<1min* 3.4M gly+4.6M EG	0.25 straws, N-vapour *1min*; **N**	*10s* **air** - *10s* wb **20**; DS 0.5M gal (120µl); *5min* 0.25M gal - PBS	66.6 (D16); 33.3 (D38)
	3	D6.5-7.5	>300	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *<1min* 3.4M gly+4.6M EG	0.25 straws, N-vapour *1min*; **N**	*10s* **air** - *10s* wb **20**; DS 0.5M gal (120µl); *5min* 0.25M gal - PBS	0.0
	22	D6	<300	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *45-60s* 3.4M gly+4.6M EG	0.25 straws, N-vapour *1min*; **N**	*10s* **air** - *10s* wb **20**; DS 0.5M gal (120µl); *5min* 0.25M gal - PBS	45.0 (D16)
	10	D7-8	>300	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *45-60s* 3.4M gly+4.6M EG	0.25 straws, N-vapour *1min*; **N**	*10s* **air** - *10s* wb **20**; DS 0.5M gal (120µl); *5min* 0.25M gal - PBS	0.0
	10	D7-8	>300	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *45-60s* 1.4M gly+6.6M EG	0.25 straws, N-vapour *1min*; **N**	*10s* **air** - *10s* wb **20**; DS 0.5M gal (120µl); *5min* 0.25M gal - PBS	0.0
	26	D6	<300	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *45-60s* 3.4M gly+4.6M EG	0.25 straws, N-vapour *1min*; **N**	*10s* **air** - *10s* wb **20**; DS 0.5M gal (180µl)	62.0 (D16)
Hudson et al. 2006	20	D6.5	<300	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *50-60s* 3.4M gly+4.6M EG	0.25 straws; N-vapour *1min*, **N**	*10s* **air** - *10s* wb **20-22**; DS 0.5M gal	70.0 (D16)
Scherzer et al. 2008	3	n.i.	805-1120	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *1min* 3.4M gly+6.6M EG	0.25 straws; N-vapour *1min*, **N**	*5s* **RT** - *15s* wb **30**; DS 0.5 M gal	0.0
	1	n.i.	1286	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *1min* 3.4M gly+6.6M EG	0.25 straws; N-vapour *1min*, **N**	*5s* **RT** - *15s* wb **30**; DS 0.5 M gal	0.0
	1	n.i.	979	*5min* 1.4M gly - *5min* 1.4M gly+3.6M EG - *1min* 3.4M gly+6.6M EG	0.25 straws; N-vapour *1min*, **N**	*5s* **RT** - *15s* wb **30**; DS 0.5 M gal	100.0 (D15); 0.0 (D28)
Campos-Chillòn et al. 2009	8	ICSI	2-8 cells	*5min* 1.5M EG - *30s* 7.0M EG+0.6M gal	OPS	1.0-0.5-0.25 M gal each *3min*; **38**	62.0 (D16)

Abbreviations in Tables 1 and 2:
AFP – anti freezing protein, Bl – blastocyst, BSA – bovine serum albumin, D – Day after ovulation (ovulation = Day0), DAPI – 4',6-diamidino-2-phenylindole, DMEM – Dulbecco's modified Eagle medium, DMSO – dimethyl sulfoxide, DT– direct transfer, eBl – early blastocyst, ER – embryo recovery, EG – ethylene glycol, exBl – expanded blastocyst, gal – galactose, gly – glycerol, H-SOF – Hepes synthetic oviductal fluid, Mo – morula, N – plunging into liquid nitrogen, n.i. – no information, n.n. – no numbers provided, PBS – phosphate buffered saline, PD – 1,2-propanediol , PR – pregnancy rate, PVP – polyvinylpyrrolidone, RT – room temperature, S – Seeding, SOF – synthetic oviduct fluid, SR – survival rate, suc – sucrose, TCM – tissue culture medium, uBl – unexpanded blastocyst, wb – water bath

Additional information by footnotes in Tables 1 and 2:
(f) birth of a foal, (1) base medium: PBS + 20% mare serum, (2) base medium: PBS + 100IU penicillin /ml + 100µg streptomycin/ml, (3) glass ampules used, not straws, (4) embedded in an agar chip, (5) trypsin pretreatment, (6) cytochalasin-B pretreatment, (7) Pyrex test tubes 13x100mm containing 0.2-0.3ml medium used, not straws, (8) glass test tubes 50x4.75 mm used, not straws, (9) 0.5 ml straws

Collecting small embryos is associated with some difficulties. Flushing on Day 6 is supposed to reduce the embryo recovery rate (*Squires* et al. 1985, *Ferreira* et al. 1997, *Castanheira* et al. 2004). However, there are also reports on high success rates for embryo collection on Day 6 (*Hochi* et al. 1995, *Carnevale* et al. 2004, *Eldridge-Panuska* et al. 2005). Induction of ovulation was shown to improve the recovery of small embryos (*Huhtinen* et al. 1997). The interval from ovulation to the arrival of equine embryos into the uterus varies from 144 to 156 hours (*Battut* et al. 1998, *Battut* et al. 2000). Differing intervals from the entry of the embryo into the uterine cavity and recovery may also contribute to the high variability of the developmental stage of embryos due to the enormous increase of embryonic growth in the uterus (*Colchen* et al. 2000). Uterine flushing performed 156 hours (6.5 days) after ovulation guarantees that the embryo has reached the uterine cavity. However, monitoring ovulation hourly does not ensure to recover a homogenous group of embryos, because morphology of equine embryos seems to depend more on the individual interval between ovulation and fertilization and individual embryonic development than on their age in relation to ovulation (*Battut* et al. 1998, *Battut* et al. 2000).

The age of the mare (*Carnevale* et al. 1993, *Meadows* et al. 1999), season (*Colchen* et al. 2000), semen quality and semen type (fresh, cooled or frozen) (*Meadows* et al. 1999, *Oberstein* et al. 2001), time of insemination (pre- or post-ovulatory) (*Carnevale* et al. 2004) and number of ovulations (*Squires* et al. 1985, *Losinno* et al. 2000) can affect embryo descent and hence recovery rate. Failure to identify the embryo, loss of the embryo due to its small size and greater specific gravity are supposed causing lower embryo recovery rate on Day 6 (*Carnevale* 2006, *Stout* 2006, *McKinnon* and *Squires* 2009). In that cases, the timing of embryo collection may have to be altered (*Carnevale* 2006).

Unique morphological characteristics might cause low survival rates after freezing large equine embryos. As the embryo grows, two distinct cell types develop: trophoblast cells lining the outer surface of the embryo, and the inner cell mass (ICM) (*Betteridge* et al. 1982, *Enders* et al. 1993). The capsule, a glycoprotein layer, forms between zona pellucida and trophoblast at the early blastocyst stage (*Betteridge* et al. 1982, *Betteridge* 1989). The blastocysts rapidly grow after the entry into the uterus, doubling their cell counts approximately every 6 hours (*Betteridge* et al. 1982, *Colchen* et al. 2000). Thus, they achieve diameters beyond 300μm usually by Day 7 after ovulation (*Colchen* et al. 2000, *Carnevale* 2006). By Day 8 after ovulation, a continuous endodermal layer is formed representing the bilaminar blastocyst (*Enders* et al. 1993).

Developmental stage influences the rate of cryoprotectant influx to the inner parts of the embryo (*Pfaff* et al. 1993, *Hochi* et al. 1994b, *Huhtinen* et al. 2000). Dehydration and equilibration with the CPA can be quantified by the extent of embryonic shrinkage. Although relative embryonic volumes were related to post-thaw viability, there was only a low predictability for the suitability of a particular embryo for cryopreservation (*Hochi* et al. 1994b). A cryoprotective agent has to penetrate the trophoblast cells first, before it reaches the ICM (*Wilson* et al. 1987). Interestingly, most extensive mitochondrial damage and degeneration of cells have been observed in the ICM (*Wilson* et al. 1987, *Barry* et al. 1989, *Bruyas* et al. 1993). The equine conceptus carries more blastomeres compared with other species (*Bruyas* et al. 1993), which might be responsible for slower equilibration with cryoptrotectants due to the lower surface-to-volume ratio in large equine embryos (*Pfaff* et al. 1993, *Barfield* et al. 2009).

Embryonic capsules thicker than 0.8μm do not allow the passage of fluids and cryoprotective agents into the embryo resulting in damage during freezing and thawing (*Legrand* et al. 2000). Lysis of the capsule by collagenase was not successful (*Tharansanit* et al. 2005), but the use of trypsin yielded pregnancies from Day 8 blastocysts (*Legrand* et al. 2000, *Legrand* et al. 2002). In mind of small numbers of embryos used in this study and the fact that *Maclellan* et al. (2002) failed to reproduce these results, the importance of the protective function of the capsule for intrauterine survival of the equine embryo has to be considered as well (*Stout* et al. 2005, *Tharasanit* et al. 2005). However, trypsin makes the capsule sticky and more prone to loss during subsequent embryo handling (*Tharasanit* et al. 2005). Thinning the capsule prevents disruption during freezing (*Tharasanit* et al. 2005), but the complete absence of the capsule has been shown detrimental to embryonic survival. Total removal of the capsule by micromanipulation or weakening to the point where it was lost during freezing-thawing process or transfer, would result in the inability to establish a viable pregnancy (*Stout* et al. 2005).

Partial removal of blastocoelic fluid and microinjection of 1.4M glycerol prior to vitrification of an expanded equine blastocyst yielded in one pregnancy on Day 15. However, ultrasonographic examination at Day 28 revealed early embryonic death (*Scherzer* et al. 2008). A method for partial laser puncture of the protein capsule of Day 8 equine blastocysts was established recently (*Wiebe* 2009, *Wiebe* et al. 2009a, *Wiebe* et al. 2009b). Although, laser treated embryos exhibited mild shrinkage after exposure to ethylene glycol, embryonic collapse could not be noticed. Treatments with laser and/or ethylene glycol did not affect large blastocysts immediately, but they showed higher rate of growth retardation during 48 hours culture period compared with controls. However, the role of the equine capsule concerning the poor ability of large equine embryos for freezing could not be clarified by now. It has also to be taken into account that the trophoblast of Day 8 blastocysts becomes bilaminar (*Enders* et al. 1993), which might represent an additional barrier for the permeation of cryoprotectants.

Intracellular cryopotectants permeate through cellular membranes because of their lower molecular weight. Common substances are glycerol, ethylene glycol (EG), 1,2-propanediol (propylene glycol) and dimethyl sulfoxide (DMSO). Extracellular, non-permeating cryoprotectants are sugars and proteins of higher molecular weight (e.g. sucrose, saccharose, galactose, serum albumin). High concentrations of cryoprotective additives cause toxic or osmotic injury to the embryo (*Leibo* 2000, *McKinnon* and *Squires* 2009).

Reports on the use of glycerol and ethylene glycol revealed inconsistent results concerning pregnancy rates after transfer of frozen-thawed equine embryos. Glycerol has been shown successful in cryopreservation of morulae and early blasto-

cysts yielding 33% pregnancy rate after transfer, but not in later developmental stages (*Meira* et al. 1993). However, glycerol-treatment resulted in a lower quality score in embryos smaller than 300µm, even if they were not frozen (*Bruyas* et al. 1993). It seemed not to completely permeate Day 6 embryos or protect mitochondrial membranes (*Wilson* et al. 1987). In the study of *Yamamoto* et al. (1982), glycerol was successful in freezing equine embryos. However, only one out of the eleven glycerol-treated, transferred embryos resulted in birth of a foal. Ethylene glycol permeates equine embryos faster and more effectively than glycerol (*Pfaff* et al. 1993). Expanded equine blastocysts survived cryopreservation after prolonged exposure to low molar EG. Unfortunately, embryos were not transferred and thus pregnancy rates not available (*O'Donovan* et al. 2000). Microscopic analysis suggested that glycerol was an effective cryoprotectant for morulae and blastocysts in contrast to ethylene glycol (*Bruyas* et al. 2000). In contrast, *Huhtinen* et al. (2000) found no differences in pregnancy rates or in percentages of DAPI-stained cells in glycerol and ethylene glycol treated small embryos.

Step-down-equilibration, the equilibration of embryos in lower concentrated successive to higher concentrated freezing media, has been shown successful with glycerol in large equine embryos (*Young* et al. 1997). In contrast, step-down-equilibration with EG seems to be toxic for embryos (*O'Donovan* et al. 2000).

Ethylene glycol and glycerol are frequently combined with sugars serving as non-permeating cryoprotectants. The addition of sucrose to EG in a 2-step freezing regimen allows the direct transfer of frozen-thawed blastocysts into recipient mares, with a pregnancy rate of 63.6% (*Hochi* et al. 1996). *Ulrich* and *Nowshari* (2002) successfully used EG and saccharose for freezing an embryo, which resulted in the birth of a life foal after direct transfer. Galactose is also used as additional CPA (*Young* et al. 1997, *O'Donovan* et al. 2000). In vitrification solutions, the combination of non-permeating cryoprotectants with ethylene glycol improved the morphology and the percentage of viable cells after thawing (*Castanheira* et al. 2004). Addition of sucrose and a dehydration step of two minutes in galactose prior to standard cryopreservation procedures did not improve the cryoprotective properties of glycerol (*Ferreira* et al. 1997, *Barfield* et al. 2009).

Further substances were tested to preserve embryos from freezing damage. Propanediol and DMSO appeared to be minimal toxic to equine embryos but they showed poor cryoprotective effects (*Seidel* et al. 1989, *Meira* et al. 1993, *Bruyas* et al. 1997a, *Bruyas* et al. 1997b, *Ferreira* et al. 1997). The use of an antifreeze protein as CPA was not effective in Day 6 embryos (*Lagneaux* et al. 1997). Agar failed to protect embryos from zona pellucida damage, but a tendency to prevent rupture was observed in larger embedded embryos (*Poitras* et al. 1994). There also might be some positive cryoprotective effects of foetal calf serum compared to polyvinylpyrrolidone in holding medium (*Huhtinen* et al. 1997). Pretreatment with cytochalasin-B resulted in a pregnancy rate of 42.9% in Day 7 embryos (*Macellan* et al. 2002). Cytochalasin-B seemed to protect larger embryos, but did not reduce the percentage of dead cells after freezing and thawing (*Thansanit* et al. 2005).

Effects of CPA removal depend on the permeability of the specific embryonic stage for the CPA and the temperature coefficient of permeability. Little is known about the relationship between the permeability to various CPAs and the temperature of the equine conceptus (*Leibo* 2000). Although, less cell death as determined by DAPI-staining was obtained if the thawing medium was supplemented with sucrose, it revealed no benefit for pregnancy rate (*Huhtinen* et al. 1997).

Conclusion

By now, only the freezing of small equine embryos revealed acceptable results. However, recovery of small embryos is less successful due to the individual embryonic descent into the uterus and differing developmental stages in relation to the time of ovulation. Further, embryos grow very fast in the uterine cavity resulting in variable diameters at a given time of collection. Hence, freezing procedures that can be modified for the requirements of an individual equine embryo dependent on size and developmental stage might increase success rates.

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