Cryopreservation of equine embryos: current state-of-the-art

Tom A.E. Stout

Utrecht University, Faculty of Veterinary Medicine, Department of Equine Sciences, Utrecht, The Netherlands

University of Pretoria, Department of Production Animal Studies, Onderstepoort, Pretoria, South Africa

Contents

During the past 15 years, embryo transfer (ET) has become increasingly widespread within the sport-horse breeding industry. At present, however, the vast majority (>95%) of horse embryos are transferred fresh or after chilled storage for up to 24 h, whereas cryopreservation is rarely employed despite its obvious potential for simplifying recipient mare management and facilitating long-term storage and international transport of embryos. A number of inter-related factors have contributed to the slow development and implementation of equine embryo cryopreservation, these include; 1) the absence of commercially-available products for reliably stimulating superovulation; 2) very poor pregnancy rates following cryopreservation of embryos >300 μm in diameter; 3) difficulty in recovering embryos at early developmental stages amenable to cryopreservation; and 4) inter-embryo variation in susceptibility to cryodamage. However, acceptable success rates (> 55% pregnancy) have been reported for both slow-frozen and vitrified small embryos (<300 μm), and there is renewed interest in cryopreservation, not only in the context of standard ET programmes, but also because it would facilitate pre-implantation genetic testing and allow wider access to techniques for producing embryos *in vitro*, such as intra-

cytoplasmic sperm injection and nuclear transfer. This article will review the current status of equine embryo cryopreservation.

Introduction

The number of equine embryo transfers performed annually worldwide has grown enormously during the last 20 years, to the point where what was once considered a highly specialized and esoteric technique is now commonplace. This growth is illustrated clearly by the International Embryo Transfer Society's (IETS) annual statistics for equine ET which, while they almost certainly underestimate true activity, document 475, 11672 and 27594 commercial equine ETs worldwide in 1999, 2004 and 2010, respectively (Thibier, 2000; 2005; Stroud, 2011). The rapid expansion in equine ET has been driven jointly by technical developments and regulatory changes. From a practical perspective, the most important breakthroughs were the development of techniques for non-surgical transfer of embryos that yielded pregnancy rates >80%, not only for freshly recovered embryos (Vogelsang et al. 1985; Riera and McDonough, 1993; McKinnon et al. 1998), but also for embryos transported at 5°C for up to 24 h (Carnevale et al. 1987; Carney et al. 1991); that embryos can be cooled and transported appreciable distances without harming pregnancy rates has made ET more accessible. The regulatory changes that have supported growth in equine ET include the relaxation, by a number of influential studbooks, of rules that had previously limited registration to one foal per genetic mother per year. Together, these changes have created an environment in which horse breeders appreciate the potential of ET for hastening genetic progress. In turn, this has led to burgeoning interest in the more advanced assisted reproductive techniques for which ET is the cornerstone. *In vitro* fertilization via intra-cytoplasmic sperm

injection, and even reproductive cloning by nuclear transfer, are now being offered as commercial services by a small number of highly specialized centres, primarily as treatments for severe sub-fertility or genetic salvage (for reviews see Allen 2005; Galli et al. 2007) .

While there has been considerable progress in equine ET, there is still room for improvement; in this respect, the major current obstacles to greater efficiency and cost-effectiveness in a commercial setting are the absence of registered products for reliably stimulating multiple ovulation, and the failure to develop techniques by which the majority of day 7 embryos can be successfully cryopreserved (Squires et al. 2003; Stout 2006). Cryopreservation has the potential to simplify ET by allowing the flushing and the transfer of embryos to be separated in both place and time. This would remove the need to synchronize recipients for every flush, thereby reducing costs, and would facilitate international distribution of valuable genetic material. Embryo cryopreservation would also allow 'temporary genetic banking', which could be advantageous to the owners of young mares from interesting bloodlines that have still to prove themselves in competition; embryos could be frozen when the mare was young and fertile, and transferred at a later date only if the mare proved its worth. A more recent development for which cryopreservation is a desirable adjunct is pre-implantation genetic diagnosis, during which a small number of cells are recovered from ET-stage embryos to test for specific heritable diseases (Choi et al. 2010); after the biopsy procedure, the embryos are ideally stored until the test results are available so that only embryos free of the genetic defect are transferred.

However, cryopreservation of horse embryos has proven problematic. While the first pregnancy from a frozen-thawed equine embryo was reported as early as 1981 (Griffin et al. 1981) the pregnancy failed at around day 60; similarly, while the

first frozen embryo foal was born the following year (Yanamoto et al. 1982), it was the only survivor from 3 pregnancies resulting from 11 transferred embryos. Although progress has been made since the early 1980s, the development of horse embryo cryopreservation has been hampered by the high costs of obtaining sufficient embryos for research, not least because reliable protocols for superovulation and IVF have also been slow to develop. Nevertheless, it is now fairly clear that, with practice, acceptable pregnancy rates (>55%) can be achieved using both conventional slow-freezing (Lascombes and Pashen 2000) and vitrification (Eldridge-Panuska et al. 2005), as long as the embryos are frozen at an early developmental stage (morula to early blastocyst), i.e. when they are less than 300 μm in diameter (Czlonkowska et al. 1985; Slade et al. 1985). Nevertheless, embryo cryopreservation has not been widely adopted in practice, as reflected by the low percentages of commercial ETs involving frozen-thawed embryos; 25/475 (5%), 408/15695 (2.6%) and 517/27594 (1.9%) in 1999, 2004 and 2010, respectively (Thibier 2000, 2005; Stroud 2011). This paper will review the current status of equine embryo cryopreservation and, in particular, some of the obstacles that have discouraged its use in clinical practice.

Freezing techniques

Two major techniques have been described for the cryopreservation of equine embryos, slow-freezing and vitrification (i.e. ultra-fast freezing).

Slow-freezing: Slow-freezing involves the gradual (i.e. stepwise) exposure of the embryo to a cryoprotective agent (CPA), prior to carefully-controlled cooling in stages. The 'classical' CPA for slow-freezing horse embryos is glycerol (10%: 1.36M) with which the embryo is equilibrated by incubation in 2 to 4 solutions of increasing concentration. Other CPAs, such as DMSO, 1,2 propanediol, ethylene

glycol (1.5M; Bruyas et al, 1995) and methanol (2.5M: Bass et al. 2004), have also been tested (for an extensive review of CPA concentrations and times of exposure tested; see Bruyas 2011), primarily because early studies suggested that exposure to glycerol, rather than freezing *per se*, was responsible for most of the damage suffered by embryos at the level of cellular ultra-structure (Wilson et al. 1987; Bruyas et al, 1995) and metabolic activity (Rieger et al. 1991). The alternative CPAs selected had a lower molecular weight which was expected to facilitate their entry into embryonic cells (Bruyas et al. 1995; Bruyas, 2011). However, while some CPAs appeared to be relatively non-toxic, e.g. ethylene glycol (Bruyas et al. 1995) and methanol (Bass et al. 2004), their cryoprotective abilities were often poor, and certainly not consistently superior to glycerol; indeed, only ethylene glycol has been reported to yield promising post-thaw pregnancy rates (7/11: Hochi et al. 1996).

During slow-freezing, the embryo is exposed to twin threats, ice-crystal formation and dehydration. As cooling proceeds, extracellular ice forms gradually leaving solutes behind, such that the osmolarity of the remaining extracellular fluid rises. To balance the intra and extra-cellular solute concentrations, water flows out of the cells (the intra-cellular fluid is not yet frozen) causing them to dehydrate. Establishing the optimal cooling rate is therefore critical since, if cooling proceeds too rapidly lethal intracellular ice crystals will form (Mazur 1977); on the other hand, if cooling is too slow, the cells will be damaged by severe dehydration and solute toxicity. This explains why slow-freezing of embryos requires a programmable freezing machine, thereby significantly increasing start-up costs, and why the straws need to be 'seeded' at -6 to -7^{o}C. Seeding initiates coordinated ice-crystal formation throughout the extra-cellular fluid with the aim of avoiding 'supercooling', i.e. cooling below the normal freezing point without ice formation. Slow-freezing is also

a time-consuming process with pre-freezing equilibration taking an average of around 40 minutes, while the freezing process itself lasts 1.5-2 h.

Vitrification: The major alternative to slow-freezing is vitrification, i.e. ultra-fast freezing to induce an instantaneous transition of both the intra- and extra-cellular fluids from the liquid to a solid, glass-like phase ("solidification"), without ice formation. Solidification is, however, only possible when very high concentrations of CPA are used (about 4-5 times higher than for slow-freezing), and when the temperature is reduced very rapidly, i.e. by direct immersion in liquid nitrogen (Liebermann et al. 2002). Plunging standard 0.25ml straws into liquid nitrogen generates a cooling rate of approximately 2500^{o}C/min; however, the freezing rate can be increased even further, to around 20000^{o}C/min, by reducing the volume of medium surrounding the embryo by stretching the straw to diminish its internal diameter (open-pulled straw technique; Vatja et al. 1998; Oberstein et al. 2001; Moussa et al. 2005), or by using cryotops or cryoloops (e.g. Oberstein et al. 2001); the accelerated cooling rate ensures rapid passage through the critical cryoinjury zones, and reduces the concentration of CPAs required. Since the first report of pregnancies from vitrified horse embryos in 1994 (Hochi et al. 1994), vitrification has attracted growing interest not only because it should theoretically avert the risk of ice crystal formation, but also because it is simpler, faster and cheaper than conventional slow-freezing; i.e. it does not require an expensive programmable freezing machine, and equilibration and freezing combined lasts less than 15 min (Eldridge-Panuska et al. 2005; Carnevale 2006). The major drawback to vitrification is that the high concentrations of CPA used are toxic to the embryo; it is therefore critical to adhere precisely to the recommended durations of immersion in the various solutions, in particular the final solution with the highest CPA concentration. This is more difficult than it sounds

because solutions with such high CPA concentrations are very dense and the embryo sinks surprisingly slowly; experience in finding and moving embryos in dense solutions is therefore a prerequisite for success with vitrification (Bruyas 2011).

Size matters

A major impediment to the implementation of embryo cryopreservation in the field is that acceptable pregnancy rates (>55%) are, at present, achievable only with embryos recovered at an early developmental stage (day 6-6.5; morula to early blastocyst) when they are less than 300 μm in diameter (Czlonkowska et al. 1985; Slade et al. 1985), i.e. very shortly after their arrival in the uterus between 144 and 168 hours (6-7 days) after ovulation (Battut et al. 1997). The influence of size appears to be even more absolute for vitrification, since embryos >300 μm show a reduced ability to re-expand during post-warming incubation (Hochi et al. 1995) and very rarely result in pregnancy after vitrification and warming (Eldridge-Panuska et al. 2005; Carnevale 2006; Scherzer et al. 2011), whereas larger embryos cryopreserved by slow-freezing do yield normal pregnancies, albeit at a lower rate (<20%) than for small embryos (Slade et al. 1985; Barfield et al. 2009).

While the obvious solution to the 'size problem' is to harvest only embryos under the 300 μm boundary, this covers a period of only a few hours immediately after the arrival of the embryo in the uterus. Moreover, the exact time of uterine entry and rate of embryo development appear to vary, depending for example on the time of year, type of semen used (fresh versus frozen) and age of the donor mare (Stout 2006). Flushing too early (e.g. day 6) risks a fall in embryo recovery (Boyle et al. 1989) because a proportion has yet to exit the oviduct, while flushing too late will result in recovery of an embryo too large to freeze successfully. The scale of the

variation in developmental rate was demonstrated by Colchen et al. (2000) who recorded ranges in diameter and cell number, respectively, of 159-365 μm and 272-2217 cells for embryos collected at 168 ± 0.5h, and 162-245 μm and 117-417 cells for embryos collected at 156 ± 0.5h after ovulation. This undoubtedly explains why Lascombes and Pashen (2000) found that 6 hourly examinations to detect ovulation, followed by embryo recovery 156 hours later, was still not sufficient to ensure that all recovered embryos were small enough to freeze (14% too large). In short, attempts to recover embryos of a size appropriate for freezing are labour intensive and by no means certain to succeed, and even though Eldridge-Panuska et al. (2005) have described a considerably less arduous system for recovering small embryos, namely by flushing exactly 8 days after the induction of ovulation with hCG (i.e. assuming ovulation 36 h after hCG administration), it is questionable whether this would be sufficiently accurate in a commercial setting given the variations in both the rate of embryo development and the exact timing of ovulation after hCG injection.

An alternative method described for recovering embryos at a stage when they are small enough to freeze successfully, is the application of PGE_2 gel to the oviduct ipsilateral to ovulation, 4 days after ovulation; this pharmacological induction of premature embryo transit through the ampullary-isthmus junction allowed Robinson et al. (2000) to recover early-stage embryos from the uterus one day later. However, the technique has not been adopted in clinical practice primarily because the PGE_2 has to be applied via a laparoscope, and the resulting need to starve the mare, surgically prepare its flank and enter the abdominal cavity greatly reduces its appeal to the owners of valuable horses (Allen 2005).

Freezing expanded blastocysts

It is clear that larger blastocysts tolerate cryopreservation less well than small embryos, both in terms of their ability to develop into viable pregnancies following transfer, and with respect to higher levels of cellular and sub-cellular damage suffered during both slow-freezing (Tharasanit et al. 2005) and vitrification (Hendriks and Stout 2010). While it is not entirely clear why larger blastocysts are more susceptible to cryodamage, contributing factors probably include the increases in embryo size and blastocoele volume (Choi et al. 2011), the very rapid increase in cell number during this period (Bruyas et al. 1995), the associated intense mitotic activity (Bruyas 2011), the presence of the blastocyst capusle (Bruyas et al. 1995: Legrand et al. 2002), and failure to tailor freezing techniques to the specific requirements of expanded blastocysts. In the case of slow freezing, for example, it is likely that the cooling rates currently employed are simply not suitable for expanded blastocysts, which may require more time than morulae or early blastocysts to reduce their intracellular water content (including blastocoele fluid) during the dehydration process that accompanies ice formation. In this respect, it is doubtless significant that an increase in size from 200 to 300 μm would be accompanied by increases in surface area and volume by factors of 2.25 and 3.375, respectively. This will significantly affect their ability to both uptake CPA and lose water during equilibration; in short, establishing more appropriate cooling rates for large blastocysts, while important, may not be straightforward.

Another attractive explanation for the difference in freezability between small and large equine embryos, is the formation of the acellular glycoprotein capsule soon after the embryo arrives in the uterus, and in association with blastocyst formation (Betteridge et al. 1982; Flood et al. 1982). A negative correlation between capsule

thickness and freezability (Legrand et al. 2002), slow permeation of glycerol and ethylene glycol through equine capsule (Gillard Kingma et al. 2011) and the good results of cryopreserving *in vitro* produced embryos (Galli et al. 2007; Campos-Chillòn et al. 2009), which do not develop a confluent capsule until after transfer to the uterus, has led to the belief that the capsule impedes access of CPAs to the embryo proper. However, attempts to improve CPA penetration by partially digesting the capsule with trypsin prior to freezing have produced conflicting results (Maclellan et al. 2002; Tharasanit et al. 2005). The explanation for the discrepancies may be that, while trypsin may thin the capsule and thereby ameliorate cytoskeleton disruption during freezing, it also renders the capsule sticky and more prone to loss during subsequent embryo handling (Tharasanit et al. 2005), where loss of the capsule is known to compromise embryo survival *in vivo* (Stout et al. 2005).

The most recent approach to improving the freezability of expanded equine embryos was arrived at serendipitously by Choi et al. (2011). In an earlier study to establish techniques for embryo biopsy for pre-implantation genetic diagnosis, the authors noted that embryo puncture and collapse did not compromise viability (Choi et al. 2010). In a follow-up study, they used a piezo drill to similarly penetrate the capsule and trophoblast, and then deliberately aspirated blastocoele fluid prior to vitrification; moreover, with one set of CPAs they achieved promising pregnancy rates (5/7: 71%) for embryos up to 650 μm (Choi et al. 2011). Although it is not known how blastocoel collapse may improve cryosurvival of expanded horse embryos, Choi et al. (2011) proposed that the most important component was the reduction in blastocoel size *per se*, rather than for example, improved access of CPA, because there was an association between post-warming survival and the extent of blastocoel collapse. In fact, improved freezability following blastocyst collapse

induced by micro-manipulation has also been described for expanded human blastocysts (Vanderzwalmen et al. 2002; Iwayama et al. 2011), and has previously been attempted with horse embryos (Scherzer et al. 2011); however, while the latter authors reported pregnancies from 4 of 9 (44%) expanded blastocysts (> 300 μm) subjected to lazer-assisted blastocoele collapse, only 1 of these embryos survived beyond day 23 of gestation. Thus, while assisted blastocoele collapse shows promise as an approach for improving the freezability of large equine embryos, it remains to be seen whether positive results, especially in terms of live foals born, can be repeated in larger trials and, indeed, whether in-field techniques for inducing blastocoele collapse can be developed that lead to similar improvements in large embryo freezability.

Using cellular damage to compare cryopreservation techniques

While the ultimate proof of the success of a cryopreservation technique is the establishment of a normal pregnancy after transfer, the fact that pregnancy is a binomial parameter (yes or no) makes it a relatively insensitive measure of differences between techniques. As a result, unfeasibly large numbers of embryos are required to generate statistically valid proof of subtle between-technique differences in the level of embryo compromise. Similarly, while assessment of morphological quality is non-damaging to the embryo, it is imprecise (4-5 quality grades) and has proven to be a misleading indicator of embryonic viability (Ferreira et al. 1997: Barfield at al. 2009). On the other hand, it is possible to obtain more precise information about damage suffered by embryos as a result of CPA exposure and/or freezing, by either culturing the embryos *in vitro* temporarily to examine their ability to re-expand and grow (Hochi et al. 1995), metabolize (Rieger et al. 1991) or repair damage (Tharasanit et al.

2005), or fixing and preparing them for analysis at the sub-cellular level. In this respect, electron microcopy has been used to demonstrate that exposure to some CPAs (e.g. glycerol) and/or freezing can damage the mitochondria and cause nuclear pycnosis, particularly within the inner cell mass (Wilson et al. 1987; Bruyas et al. 1993, 1995, 2000), accompanied by damage to other cytoplasmic organelles and structures (e.g. endoplasmic reticulum, golgi apparatus, microvilli) and vacuolation of the cytoplasm (Landam e Alvarenga et al. 1993; Ferreira et al. 1997); many of these changes could be ameliorated by using a CPA with lower molecular weight, although as previously discussed, few of the potential alternative CPAs have adequate cryoprotective abilities (Bruyas et al. 1995). Rieger et al. (1991) used radiolabelled glucose and glutamine to demonstrate that CPA exposure affected embryonic metabolic activity, while more recent studies have used specific fluorescent stains and confocal microscopy to demonstrate that cryopreservation leads to a loss of membrane integrity (Tharasanit et al. 2005; Moussa et al, 2005) in a proportion of cells, and can result in irreversible disruption of the cytoskeleton (Tharasanit et al. 2005). While there are differences between published studies in estimates of how much damage is caused by exposure to various CPAs or the cryopreservation process *per se*, some of these differences undoubtedly relate to the varying lengths of time for which embryos were incubated post-thaw before being fixed for analysis. Moreover, these studies have revealed types of damage and uneven distributions of the damage (predeliction for ICM cells: Wilson et al. 1987; Bruyas et al. 1993) that may explain why embryos that appear to be morphologically normal post-thaw are no longer viable. Assessing the degree of cellular disruption or metabolic compromise may therefore be a useful aid in the selection of the least toxic CPAs (particularly relevant for vitrification media) and the most suitable freezing techniques or cooling rates for

specific classes of embryo. Admittedly however, it is not yet clear to what degree various levels and types of ultrastructural damage compromise the viability of the embryo as a whole.

Conclusions

Embryo cryopreservation is a technique with considerable potential for improving the flexibility and reducing the costs of equine embryo transfer. Moreover, cryopreservation of small embryos by either slow-freezing or vitrification is, given adequate operator experience, reasonably successful. The most significant remaining impediment to the more widespread adoption of cryopreservation in practice is thus the difficulty in ensuring that embryos are collected after their entry into the uterine cavity, but before expansion to beyond 300 μm in diameter. On the other hand, it must be hoped that induction of blastocoele collapse, or other novel techniques, will prove to be reliable methods for extending the size range of embryos that can be cryopreserved with success, and that new techniques for examining damage to cell architecture and function will help identify the most appropriate techniques for cryopreserving horse embryos of all sizes.

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