Effect of cryopreservation on the cellular integrity of equine embryos

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Abstract

Horse embryos are rarely cryopreserved in practice because expanded blastocysts tolerate freezing poorly, and the embryo begins expanding very soon after entering the uterine cavity. This study examined the effects of freezing on cytoskeleton integrity, and investigated whether cell damage could be reduced using trypsin to thin the blastocyst capsule or cytochalasin-B (cyto-B) to stabilise the cytoskeleton. Sixty-nine embryos were recovered 7 days after ovulation and equilibrated in 10% glycerol, with or without pretreatment with 0.2% trypsin or 7.5 µg/ml cyto-B. Forty-two of the embryos were frozen; the rest were used to determine whether pre-freezing treatment alone caused cell damage. Subsequently, embryos were stained with 4',6-diamidino-2-phenylindole dihydrochloride, to identify dead cells, and fluorescently labelled phalloidin, to assess cytoskeleton quality. Without freezing, none of the treatments affected cell viability. And although Cyto-B altered actin distribution, the cytoskeleton returned to normal during a 4-h culture. Following cryopreservation, the percentage of dead cells ($11.1 \pm 1.3\%$) did not differ between treatments ($P > 0.05$), but significantly fewer cells died in small ($\leq 300 \mu\text{m}$) than in large embryos when neither pretreatment was used ($P > 0.05$); the effect of embryo size was, however, not significant after pretreatment with trypsin or cyto-B, and trypsin improved the likelihood of an intact cytoskeleton post thaw. However, trypsin treatment also resulted in a 'sticky' capsule that complicated embryo handling, and cyto-B-induced actin-depolymerisation was not reversed during a 6-h post-thaw incubation. Thus, while trypsin pretreatment improved cytoskeleton preservation and both trypsin and cyto-B may reduce cell death during cryopreservation of large embryos, both treatments induced other changes likely to compromise embryo survival.

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Introduction

During the past decade, embryo transfer (ET) has become increasingly widespread within the sport-horse breeding industry; pregnancy rates of 50–80% are now commonly achieved after non-surgical transfer of embryos either directly after recovery (Vogelsang *et al.* 1985, Meira *et al.* 1993, Riera & McDonough, 1993, McKinnon *et al.* 1998) or after storage at 5°C for up to 24 h (Carnevale *et al.* 1987, Carney *et al.* 1991). However, the commercial exploitation of equine ET has been restricted by the limited success of embryo cryopreservation and the high costs and moderate success of superovulation (Squires *et al.* 2003); the need to synchronise multiple recipient mares for a mean of only 0.7 embryos per flush increases the costs dramatically (Palmer & Jousset 1975, Squires *et al.* 1999).

Acceptable pregnancy rates can be achieved with frozen-thawed horse embryos (50–60%: Skidmore *et al.* 1991, Lascombes & Pashen 2000) only if the embryos are

frozen at an early developmental stage (day 6–6.5; morula to early blastocyst) when they are less than 250 µm in diameter (Slade *et al.* 1985), i.e. shortly after their arrival in the uterus at around day 6 after ovulation (Battut *et al.* 1997). Unfortunately, the precise time of uterine entry and rate of embryo development differ depending on the time of year, type of semen used (fresh vs frozen) and mare age (Meadows *et al.* 1999).

The reason why larger equine blastocysts do not tolerate cryopreservation is not clear, but it has been proposed that it involves the acellular capsule that forms around the embryo during blastulation (Betteridge *et al.* 1982, Flood *et al.* 1982). A negative correlation between capsule thickness and freezability has led to the suggestion that the capsule may impede access of cryoprotectants to the embryo proper (Legrand *et al.* 1999, Bruyas *et al.* 2000). Indeed, partially digesting the capsule by immersing expanded blastocysts in a trypsin solution prior to freezing resulted in an unusually high pregnancy rate after subsequent thawing and transfer to recipient mares in one

report (6/8: Legrand *et al.* 2000), but not others (3/11: Legrand *et al.* 2002, 0/14: Maclellan *et al.* 2002). The probable cause of reduced embryo viability during freezing and thawing is the disruption of cell organelles, in particular the cytoskeleton, as a result of intracellular ice formation (Wilson *et al.* 1987, Dobrinsky 1996). Cytochalasin B (cyto-B) is a reversible inhibitor of actin polymerisation which, by temporarily deconstructing the actin filaments, has been shown to reduce cytoskeleton damage during cryopreservation and, thereby, improve the viability of frozen-thawed porcine embryos (Dobrinsky *et al.* 2000). Maclellan *et al.* (2002) reported that expanded horse blastocysts treated with cyto-B prior to cryopreservation subsequently yielded pregnancies (3/7) at a similar rate to embryos frozen using a conventional glycerol protocol (4/7), and contained similar levels of dead cells; they did not examine the effect of cyto-B on embryonic cytoarchitecture. Moreover, while the effects of cryopreserving equine embryos on the microstructure of some cellular organelles (e.g. mitochondria; Wilson *et al.* 1987, Bruyas *et al.* 1993, Ferreira *et al.* 1997) has been examined, the conclusions with regard to the degree of damage and proportion attributable to cryoprotectant are conflicting, and the effect on the cytoskeleton has not been reported. The aims of the current study were to investigate the level of cytoskeleton disruption suffered by horse embryos during freezing and thawing using glycerol as cryoprotectant, and to examine whether additional pre-freezing treatment with trypsin to thin the capsule or cyto-B to temporarily depolymerise the cytoskeleton would improve post-thaw cell viability and actin cytoskeleton integrity.

Materials and Methods

Embryo production, collection and pre-freezing examination

Sixty-nine embryos were recovered from Dutch Warm-blood mares 7 days after an ovulation was detected during daily ultrasonographic examination of the ovaries; embryos were thus 7.5 ± 0.5 days old at recovery. During the preceding oestrus and beginning when the dominant follicle(s) exceeded 35 mm in diameter, mares had been inseminated every second day with $300\text{--}500 \times 10^6$ progressively motile sperm from a stallion of proven fertility. Embryos were collected by non-surgical uterine lavage using 3L Dulbecco's phosphate-buffered saline (PBS) supplemented with 0.5% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich Chemicals BV, Zwijndrecht, The Netherlands). Immediately after recovery, the embryos were examined using a stereoscopic dissecting microscope and assigned a quality score of 1–5 (1 = excellent, 5 = degenerate or dead; McKinnon & Squires 1988). Only grade 1 and 2 embryos (excellent and good) were used in this experiment, and these were washed and then transported to the laboratory at room temperature ($\sim 25^\circ\text{C}$) in ovum culture medium (OCM; ICN Biomedicals, Zoetermeer, The Netherlands).

Upon arrival at the laboratory and within 1 h of collection, the diameter of each embryo, excluding its zona pellucida and/or capsule, was measured using a microscope equipped with an eyepiece micrometer. Next, the embryos were washed using PBS (pH 7.2; Gibco BRL, Paisley, Strathclyde, UK) and stained with $0.1\text{ }\mu\text{g/ml}$ of the non-toxic, fluorescent membrane-impermeable DNA stain 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes Europe BV, Leiden, The Netherlands) in PBS at 37°C to identify any dead cells, as described by Huhtinen *et al.* (1995). Labelled embryos were examined using a conventional fluorescent microscope (BH2-RFCA; Olympus, Tokyo, Japan) equipped with a digital camera (Coolpix990; Nikon Instruments Europe B.V., Badoedorp, The Netherlands); images were recorded after exposing the embryos to u.v. light for approximately 30 s.

Experiment I: The effect of pre-freezing treatments on cellular and cytoskeletal integrity

Seventeen embryos were used to study the effect on cell viability and actin cytoskeleton quality of treatment with cryoprotectant (glycerol) alone or in combination with trypsin or cyto-B, without subsequent freezing and thawing. Four embryos were subjected to a standard pre-freezing, four-step, 10% glycerol equilibration procedure, i.e. 10 min each in 2.5, 5, 7.5 and 10% glycerol in PBS at room temperature (25°C). A further five embryos were immersed in 0.2% (w/v) trypsin in PBS (Sigma-Aldrich Chemicals BV) for 15 min at 37°C (as described by Legrand *et al.* 2000) before equilibration in the glycerol solutions, and the remaining eight embryos were equilibrated in glycerol solutions containing $7.5\text{ }\mu\text{g/ml}$ cyto-B (dissolved in dimethylsulphoxide; both Sigma-Aldrich Chemicals BV). After the 10-min incubation in 10% glycerol, the cryoprotectant and cyto-B were washed out using a reversed four-step equilibration in PBS containing decreasing glycerol concentrations (10 min each at 7.5, 5, 2.5 and 0%). After the final wash, the embryos were restained with DAPI so that dead cells could again be counted. Thereafter, embryos were fixed overnight in 4% paraformaldehyde and stored in PBS at 4°C until further analysis. Of the eight cyto-B-treated embryos, two were stained and fixed immediately after removal of the cryoprotectant while the remaining six were first incubated in OCM at 37°C for either 2 h ($n=2$) or 4 h ($n=4$). An additional ten embryos were fixed without either freezing or any other treatment, to establish 'normal' percentages of dead cells and patterns of actin distribution.

Experiment II: Effect of cryopreservation on cell viability and cytoarchitecture

After DAPI staining to count the number of dead cells, 42 embryos were assigned randomly to one of the three freezing protocols outlined above: 14 embryos using the glycerol-alone protocol, 11 after pretreatment with trypsin and 17 in glycerol solutions containing cyto-B. Six of the

cyto-B embryos were assigned to a further 6-h culture post thaw to examine whether the effects of cyto-B on the actin cytoskeleton could be reversed after freezing and thawing. The ten untreated, unfrozen embryos described in experiment I were again used as the control group.

Freezing and thawing protocols

Cryopreservation was performed essentially as described by Czlonkowska *et al.* (1985). Briefly, the equilibrated embryo was loaded into a 0.25 ml polyvinyl chloride straw (IMV Technologies, L'Aigle, France) in a droplet of 10% glycerol solution sandwiched between two air bubbles. The straw was sealed with a straw plug and placed vertically in a programmable controlled-rate freezing machine (Planer Kryo 10 Series II, Sunbury-on-Thames, UK). Initial cooling was at 1 °C/min from ambient temperature down to -6 °C, at which temperature the straws were held for 5 min and 'seeded' by running metal forceps precooled in liquid nitrogen along them. After seeding, cooling was continued at 0.3 °C/min down to -33 °C, where the straws were held for 10 min before being plunged into liquid nitrogen. All embryos were stored in liquid nitrogen for at least 1 week before thawing for further examination.

The straws were thawed by holding them in the air for 10 s and then submerging them in 35 °C water for 1 min. Next, the embryo was expelled into a PBS solution containing 10% glycerol and 0.25 M sucrose (Sigma-Aldrich Chemicals BV). The cryoprotectant was then washed out by incubating the embryos for 10 min each in solutions of 0.25 M sucrose in PBS containing decreasing concentrations of glycerol (7.5, 5, 2.5 and 0%). Finally, the embryos were restained with DAPI to allow the number of dead cells to be counted, fixed overnight in 4% paraformaldehyde and stored in PBS at 4 °C until analysis of total cell number and cytoskeleton quality.

Assessing actin cytoskeleton integrity

To visualise the actin cytoskeleton, embryos were permeabilised by immersion in 0.1% Triton X-100 in PBS for 10 min, and then stained for 1 h at room temperature with a 15 µg/ml solution of Alexa Fluor 488-phalloidin (Molecular Probes Europe BV) in PBS. After washing in PBS, the embryos were counterstained with 0.1 µg/ml DAPI to count cell nuclei. To localise the cell plasma membrane, a number of embryos were labelled for 1.5 h at room temperature with a 5 µg/ml solution of a wheat germ agglutinin (WGA)-Alexa Fluor 594 conjugate (Molecular Probes Europe BV) in PBS. Labelled embryos were mounted on glass microscope slides with an antifade medium to retard photobleaching (vectashield; Vector Laboratories, Burlingame, CA, USA), sealed under a coverslip using nail polish and stored in the dark at room temperature until they could be analysed using either a Leica TCS SP confocal laser-scanning microscope or a Bio-Rad Radiance 2100MP confocal and multiphoton system (Bio-Rad,

Hemel-Hempstead, UK) mounted on a Nikon TE300 inverted microscope (Uvikon, Bunnik, The Netherlands). A combination of a 488-argon ion and a 543-helium neon laser was used to produce sequential optical scans of the Alexa Fluor 488-phalloidin-labelled actin cytoskeleton and the WGA-Alexa Fluor 594-labelled plasma membrane respectively. The DAPI-stained nuclear material was excited using a 100 fs pulsed 780 nm excitation laser source (a mode-locked titanium:sapphire laser; Tsunami; Spectra Physics, Mountain View, CA, USA). The images produced by sequential scans via the different colour channels were then merged and recorded in digital format. Subsequently, the images were displayed using Adobe Photoshop 7 software (Adobe Systems Inc., Mountain View, CA, USA) and the appearance of the actin cytoskeleton was assessed. Following cytoskeleton assessment, the embryos were flattened by gently pressing on the coverslip and the total number of cells in each embryo was counted, using a conventional fluorescent microscope equipped with an eyepiece counting grid, for calculation of the percentage of dead cells.

Statistical analysis

The percentage of cells that died during freezing and thawing was calculated as the increase in the number of DAPI-positive cells divided by the total cell number. Pre-freezing embryo diameter and the extent of freezing-induced cell death were compared between experimental groups using one-way ANOVAs with a Bonferroni correction. Non-parametric tests (Kruskal-Wallis and Mann-Whitney U) were used to compare post-thaw actin cytoskeleton quality. In all cases, the statistics were performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA), and differences were considered significant when $P < 0.05$.

Results

Actin cytoskeleton quality in horse embryos

Horse embryos fixed and stained immediately after collection were typified by intense actin staining precisely and evenly organized within the cell cortex, such that the cell borders were sharply demarcated; this pattern was used as a standard for 'good' actin cytoskeleton morphology (grade I: Fig. 1A). The appearance of the actin cytoskeleton changed when embryos were treated with cyto-B or frozen and thawed, and the extent of this cytoskeleton disruption was accounted for by the creation of a further two quality grades. A grade II actin cytoskeleton was characterised by large areas where the cell borders, although grossly maintained, were indistinct and stained only patchily, and where small clumps of actin were visible within the cytoplasm (Fig. 1B). A grade III cytoskeleton had large areas where the cell borders lacked obvious actin staining and where, instead, most of the visible actin was in the form of intracytoplasmic clumps (Fig. 1C).

Figure 1 Photomicrographs to illustrate the actin cytoskeleton quality scoring system (grades I–III) developed for horse embryos examined using a multiphoton laser-scanning microscope. The embryos were stained with Alexa Fluor 488-phalloidin (green) to visualise actin filaments and DAPI to stain the cell nuclei (blue). (A) A grade I cytoskeleton was typified by precise, sharp restriction of actin staining to the cell borders. (B) Grade II was characterised by a less distinct outlining of the cells combined with occasional small clumps of actin in the cytoplasm and (C) a grade III actin cytoskeleton had large areas lacking actin staining, with the visible actin largely agglomerated in intracytoplasmic clumps. Scale bars represent 100 μ m (A and B) and 50 μ m (C).

Experiment I

The 17 embryos used to examine the effect of pre-freezing treatment (glycerol $\pm$ trypsin or cyto-B) on cell viability and cytoskeleton structure contained a mean $\pm$ S.E.M. of $1\,948 \pm 612$ cells. The mean percentage of cells dead at embryo recovery was $0.1 \pm 0.0\%$, and this figure was not increased by any of the pre-freezing treatments (Table 1). All of the embryos that had been exposed to glycerol alone or trypsin followed by glycerol were recorded to have a grade I actin cytoskeleton following washing to remove the glycerol (Figs 2 and 3A and B). By contrast, the actin cytoskeleton changed dramatically in embryos incubated in glycerol solutions containing 7.5 μ g/ml cyto-B. In general, cyto-B treatment resulted in a lower intensity of actin labelling at the cell cortex and the appearance of patches of actin staining within the cytoplasm (i.e. a grade II cytoskeleton; Fig. 3C). With regard to the reversibility of the cyto-B effect, the actin cytoskeleton did not repolymerise during a subsequent 2-h incubation in cyto-B-free culture medium (Fig. 3D), but incubation for 4 h was sufficient to allow recovery of a ‘normal’ grade I actin cytoskeleton pattern (Figs 2 and 3E).

Experiment II

Mean $\pm$ S.E.M. embryo diameter ($382 \pm 25.6 \mu$ m; range 160–1000 μ m), total cell number (1591 ± 226) and percentage of dead cells (0.1 ± 0.0) prior to freezing did not differ between the experimental groups. However, the number of embryos that fractured during thawing did tend

to differ between treatments (2/14 conventionally frozen, 3/11 trypsin-treated, 0/17 cyto-B-treated; $P = 0.09$); only the actin cytoskeleton quality of intact areas of the fractured embryos could subsequently be analysed meaningfully.

The mean percentage of dead cells following freezing and thawing did not differ significantly between the conventional, trypsin- and cyto-B-treated groups (14.6 ± 2.4 , 9.6 ± 2.8 and $9.7 \pm 2.4\%$ respectively; Table 2). However, the percentages of dead cells post thaw were significantly higher than in control, non-frozen embryos ($0.15 \pm 0.6\%$; $P < 0.001$).

With regard to the effect of embryo size on freezability, embryos $>300 \mu$ m in diameter had a significantly higher percentage of dead cells post thaw than those $\leq 300 \mu$ m if they were frozen without additional trypsin or cyto-B treatment (19.0 ± 2.9 vs $8.5 \pm 2.1\%$; $P < 0.05$).

On the other hand, the effect of blastocyst size on freezing-induced cell death was not statistically significant if embryos were pretreated with either trypsin (13.8 ± 4.9 vs $5.4 \pm 0.7\%$; $P = 0.14$) or cyto-B (12.3 ± 5.5 vs $8.1 \pm 2.3\%$; $P = 0.32$; Table 2), primarily because the percentage of dead cells in larger embryos tended to be lower in these groups. As for the actin cytoskeleton, eight of the ten (80%) non-frozen control embryos had a grade I cytoskeleton (Figs 1A and 4), and freezing and thawing led to a dramatic drop in mean cytoskeleton quality. Mean cytoskeleton quality also tended to differ between freezing protocols ($P = 0.058$); in particular, the number of embryos that retained a grade 1 cytoskeleton post thaw

Table 1 The average number of cells and percentages that were dead in horse embryos at recovery and after treatment with glycerol $\pm$ trypsin or cyto-B, without cryopreservation. Values are means $\pm$ S.E.M.

Pre-freezing treatment (number of embryos)	Total cells per embryo	Average % dead cells	
		At recovery	After treatment
Glycerol (4)	1948 $\pm$ 612	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
Trypsin + glycerol (5)	1562 $\pm$ 602	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1
Cyto-B + 0–2 h of culture (4)	1185 $\pm$ 256.1	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1
Cyto-B + 4 h of culture (4)	999 $\pm$ 241	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0

Figure 2 Actin cytoskeleton quality (grades I–III) in horse embryos immediately after collection (controls) or after glycerol, trypsin or cyto-B pre-treatment, without cryopreservation. Solid bars = grade I; open bars = grade II. The numbers of embryos in each group is noted above each column. *Cyto-B treatment significantly reduced mean cytoskeleton quality ($P < 0.05$).

was significantly higher in trypsin-treated (4 of 11) than in the conventionally frozen (0 of 14) or cyto-B-treated (0 of 17) groups ($P = 0.002$; Fig. 3J). Immediately post thaw, all of the frozen-thawed cyto-B-treated embryos had a grade II actin cytoskeleton pattern (Fig. 3K), similar to that described in experiment I for embryos treated with cyto-B without freezing and thawing. In this case, however, a 6-h post-thaw incubation in cyto-B-free medium was not sufficient to return the actin cytoskeleton to a grade I appearance (Fig. 3L).

The fluorescently labelled lectin, WGA, was used to verify the location of the plasma membrane with respect to the actin cytoskeleton (Fig. 3F–H) and to determine whether disruption of the latter during cryopreservation necessarily correlated with loss of plasma membrane integrity. The dual staining revealed that actin cytoskeleton disruption, typified by dispersal of actin labelling from the cell borders, often occurred in the absence of any disruption of the adjacent plasma membrane (Fig. 3I).

Discussion

The success of equine embryo cryopreservation depends critically on embryo size and developmental stage (Slade *et al.* 1985, Skidmore *et al.* 1991), but recovering early embryos likely to survive freezing and thawing is labour intensive, and complicated by variations in the rate of embryo development. This problem could be circumvented by treatments to hasten embryonic descent into the uterus (e.g. applying prostaglandin E2 to the oviduct; Robinson *et al.* 2000) or techniques to successfully cryopreserve expanded blastocysts. With regard to the latter, it would help to first establish the type and extent of damage

suffered by expanded blastocysts during cryopreservation, and then to determine which indices of damage correlate with embryo survival after transfer. In the current study, freezing and thawing horse embryos using glycerol as cryoprotectant resulted in disruption of the cytoskeleton and appreciable cell death, particularly in large ($>300\mu\text{m}$) embryos. The higher rate of cell death in larger embryos (19 vs 8.5%) probably explains their reduced viability; previous studies have demonstrated that inner cell mass cells are more susceptible than trophectoderm cells to freezing-induced damage (Wilson *et al.* 1987, Bruyas *et al.* 1993, 1995, 2000). As dramatic as the cell death, however, was the freezing-induced disruption of the actin cytoskeleton within an intact cell plasma membrane. Irreversible cytoskeleton disruption would render the affected cell non-viable and, if it occurred on a wide scale, the embryo non-viable because a normal cytoskeleton is critical to cell function and embryo development (Barnett *et al.* 1997, Matsumoto *et al.* 1998, Wang *et al.* 2000). Indeed, because the cytoskeleton supports the cell plasma membrane and maintains intracellular organelle organisation (Boldogh *et al.* 1998, Valderrama *et al.* 1998), cytoskeleton disruption probably explains most of the freezing-induced changes in cell morphology and organelle distribution described previously for cryopreserved horse embryos (Ferreira *et al.* 1997).

In the current study, cell damage was predominantly an effect of freezing and thawing *per se* rather than of glycerol toxicity; this broadly supports the findings of Wilson *et al.* (1987) who reported consistent mitochondrial damage in embryos cryopreserved in 10% glycerol but only sporadic abnormalities in embryos exposed to glycerol without freezing. That other authors arrived at the contradictory conclusion that glycerol toxicity is the more significant fac-

Table 2 Mean $\pm$ S.E.M. embryo diameter prior to treatment, and cell number and percentage of dead cells after treatment with glycerol, with or without trypsin or cyto-B, and freezing and thawing.

Treatment	Group (no. of embryos)	Diameter (μ m)	Total cell number	% dead cells
Non-treated controls	$\leq 300 \mu$ m (5)	244 $\pm$ 19.4	648 $\pm$ 226	0.2 $\pm$ 0.1
	$> 300 \mu$ m (5)	460 $\pm$ 78.0	2285 $\pm$ 772	0.1 $\pm$ 0.0
Conventional freezing	$\leq 300 \mu$ m (5)	244 $\pm$ 17.2	731 $\pm$ 129	8.5 $\pm$ 2.1 ^a
	$> 300 \mu$ m (7)	445 $\pm$ 31.7	2161 $\pm$ 701	19.0 $\pm$ 2.9 ^b
Trypsin pretreatment	$\leq 300 \mu$ m (4)	235 $\pm$ 29.9	445 $\pm$ 95	5.4 $\pm$ 0.7
	$> 300 \mu$ m (4)	565 $\pm$ 134.2	2861 $\pm$ 1052	13.8 $\pm$ 4.9
Cyto-B pretreatment	$\leq 300 \mu$ m (4)	225 $\pm$ 18.9	483 $\pm$ 202	8.1 $\pm$ 2.3
	$> 300 \mu$ m (7)	553 $\pm$ 82.7	3292 $\pm$ 935	12.3 $\pm$ 5.5
Cyto-B + 6-h post-thaw incubation	$\leq 300 \mu$ m (3)	186.7 $\pm$ 6.7	290 $\pm$ 40	6.3 $\pm$ 1.5
	$> 300 \mu$ m (3)	433 $\pm$ 59.2	757 $\pm$ 108	11.7 $\pm$ 1.3

^{a,b}Within a column, different superscripts denote values that differ significantly within a treatment ($P < 0.05$)

tor (Rieger *et al.* 1991, Bruyas *et al.* 1993, 1995) appears to be primarily a result of marked between-study differences in the duration of incubation post thaw and the indices of cell death employed. Bruyas and co-workers categorised cells with electron microscopically detectable nuclear pycnosis after 6-h incubation *in vitro* as dead (22–54% of cells; Rieger *et al.* 1991, Bruyas *et al.* 1993, 1995, 2000); we counted cells with a permeable plasma membrane soon after glycerol removal as dead (5–19% of cells). Since a shorter culture (1 h) resulted in lower rates of cells classified as dead via electron microscopy (Wilson *et al.* 1987, Bruyas *et al.* 1993), and improved culture conditions dramatically reduced pycnosis (to 2–3%; Bruyas *et al.* 2000), it appears that the toxic effects of glycerol were exaggerated by the culture system used in earlier studies. Overall, it appears that glycerol may induce mild damage at the level of mitochondria that progresses to affect cell viability only if the embryo is forced to develop further in a suboptimal environment. Other factors likely to affect the apparent severity of glycerol toxicity include the equilibration procedure and the size/developmental stage of the embryo. In particular, relatively slow addition of glycerol (e.g. 4×10 -min steps) is thought to increase the final intracellular glycerol concentration and thereby reduce osmotic cell damage (Bruyas *et al.* 2000); the fewer, shorter glycerol equilibration steps used in earlier studies may thus have exacerbated cell damage. With regard to embryo size, while most studies examined glycerol toxicity in relatively

small day-6 embryos, we concentrated on day-7 expanded blastocysts more likely to sustain damage during freezing. This may have influenced the susceptibility to glycerol toxicity since Legrand *et al.* (2000) recently reported that blastocysts with a thick capsule suffer little cell death during equilibration in glycerol, possibly because the capsule completely blocks glycerol and fluid movements and thereby prevents cryoprotectant-induced osmotic cell damage. However, since these authors also report that a moderately thick capsule leads to the highest rates of cryoprotectant-induced cell death (they propose that in this situation water can exit but glycerol cannot enter), it must be assumed that trypsin treatment increases the permeability of the capsule to glycerol more dramatically than would be possible by a straightforward partial reduction in thickness.

The only pre-freezing treatment that noticeably affected embryo quality in the current study was cyto-B which, as expected, led to dispersal of actin from the cell cortex. Unexpectedly, the pattern of actin depolymerisation observed in cyto-B-treated horse embryos was very different from the complete disappearance of staining reported for similarly treated pig embryos (Dobrynsky *et al.* 2000). This suggests either that the cyto-B treatment used in the current study did not induce complete actin depolymerisation, or that depolymerisation takes different forms in these two species. Nevertheless, culturing cyto-B-treated embryos for 4 h in cyto-B-free medium allowed recovery

Figure 3 Photomicrographs to illustrate the actin cytoskeleton morphology of fresh and frozen-thawed equine embryos cryopreserved after different pre-freezing treatments. Embryos were stained with Alexa Fluor 488-phalloidin (green) to visualise the actin cytoskeleton, DAPI for the cell nuclei (blue) and WGA to label the lectin-reactive elements in the plasma membrane (red), and then examined using a multiphoton excitation microscope. Scale bars represent 100 μ m (A, D, E and J) and 50 μ m (B–C, F–I and K–L). (A to C) The actin cytoskeleton of non-frozen embryos treated with (A) glycerol, (B) glycerol and trypsin or (C) glycerol and cyto-B. Panel (A) has been additionally stained with WGA, and the surrounding red layer is the lectin-containing blastocyst capsule. (D and E) Cyto-B-treated embryos were incubated for an extra (D) 2 h or (E) 4 h in cyto-B-free medium; a 4-h incubation was sufficient for complete actin repolymerisation. (F to G) Triple staining of actin (green), cell nuclei (blue) and WGA-reactive lectins in the plasma membrane and blastocyst capsule (red) of a control (non-frozen) embryo. Panels (F and G) respectively show staining with WGA or phalloidin separately; panel (H) shows the superimposition of the two, and thereby demonstrates that WGA and phalloidin staining were colocalised at the cell borders. (I) Embryo frozen after trypsin pretreatment and stained with WGA and phalloidin. The white arrows show the WGA-labelled outline of the intact plasma membrane in areas where the actin cytoskeleton has been disrupted. (J) Grade I actin cytoskeleton in an embryo frozen and thawed after trypsin pretreatment. (K and L) Actin cytoskeleton of frozen-thawed embryos pretreated with cyto-B and either (K) fixed immediately after thawing and removal of glycerol or (L) following an extra 6-h incubation in OCM in an attempt to reverse the actin depolymerisation.

Figure 4 Actin cytoskeleton quality of horse embryos frozen in 10% glycerol following different pre-freezing treatments (CF = conventional slow freezing). Solid bars = grade I; grey bars = grade II; open bars = grade III. The number of embryos in each group is noted above each column. *Trypsin pretreatment significantly increased the likelihood of a grade I cytoskeleton post thaw ($P = 0.02$).

of a grade I cytoskeleton, thereby demonstrating that the depolymerisation was reversible.

Freezing and thawing disrupted the actin cytoskeleton and induced cell death in all treatment groups, but small embryos ($<300\ \mu\text{m}$) tended to suffer less damage. In fact, the effect of size only reached statistical significance in embryos frozen using the standard glycerol protocol, primarily because large embryos in this group tended to suffer more cell death than in others; this suggests that trypsin and cyto-B may preferentially protect larger embryos. In the case of trypsin, this supports the finding of Legrand *et al.* (1999) that partial capsule digestion reduces cell death during cryopreservation. In addition, because in the current study only embryos pretreated with trypsin maintained a grade I cytoskeleton post thaw, it appears that thinning the capsule prevents cytoskeleton disruption during freezing. However, trypsin treatment also resulted in a 'stickier', more difficult to handle capsule and, since the capsule is essential for conceptus survival *in vivo* (Stout *et al.* 2005), it is possible that the poor pregnancy rates recorded in recent studies with trypsin-treated frozen-thawed blastocysts (Legrand *et al.* 2002, Maclellan *et al.* 2002) were a result of loss of the 'sticky' capsule during ET. Cyto-B has been proposed to reduce cell damage during embryo freezing or manipulation by depolymerising actin filaments and thereby increasing the flexibility of cell plasma membranes (Huhtinen *et al.* 1995); this may be why cyto-B-treated embryos in the current study tended to be less likely to fracture during thawing. In the case of pig embryos, cyto-B treatment prior to vitrification reduced cytoskeleton disruption and improved survival of expanded blastocysts, but not morulae or early blastocysts (Dobrinsky *et al.* 2000). The results

of the current study similarly suggest that any protective effects of cyto-B during the cryopreservation of horse embryos are preferential to large blastocysts. On the other hand, the effects of cyto-B on the cytoskeleton could not be reversed during a 6-h post-thaw incubation, whereas in non-frozen embryos the cytoskeleton repolymerised within 4 h. The failure, or delay, of cytoskeleton regeneration post thaw could be because freezing critically damaged the cytoskeleton or its reassembly mechanism. In this respect, cytoskeleton regeneration is powered by mitochondria (Ahn *et al.* 2002), and mitochondrial damage during freezing (Wilson *et al.* 1987) might have delayed or prevented this process. Nevertheless, since Maclellan *et al.* (2002) were able to establish pregnancies with frozen-thawed cyto-B-treated horse embryos, cytoskeleton recovery must be possible after freezing and thawing and, as proposed for recovery from glycerol toxicity, it could be that any damage is more readily compensated *in vivo* than *in vitro*.

In conclusion, the current study used fluorescent stains to demonstrate that cryopreserving horse embryos leads to cytoskeleton disruption and cell death. Equilibration in glycerol, with or without cyto-B or trypsin, had no detectable lasting detrimental effects on cell architecture or viability. Trypsin pretreatment significantly improved the likelihood of an embryo maintaining an intact cytoskeleton during freezing, but further evidence that trypsin or cyto-B protected expanded blastocysts during cryopreservation was marginal. Moreover, questions remain about the suitability of these treatments because trypsin alters the physical characteristics of the blastocyst capsule, and cyto-B-induced actin depolymerisation is difficult to reverse after thawing. Nevertheless, cytoskeleton structure

post thaw may be a valuable parameter for embryo quality and, as more fluorescent markers for intracellular structure and function become available, it is proposed that this approach will become an increasingly useful and sensitive adjunct to ET for evaluating the suitability of various freezing techniques.

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