Quantitative analysis of morphological modifications of day 6.5 horse embryos after cryopreservation: differential effects on inner cell mass and trophoblast cells

J-F. Bruyas¹, J. Bézard², D. Lagneaux² and E. Palmer²

¹Service Pathologie de la Reproduction, Ecole Nationale Vétérinaire de Nantes, CP 3013, 44087 Nantes Cedex 03, France; and ²INRA Station de Physiologie de la Reproduction, Centre de recherche de Tours, 37380 Nouzilly, France

Sixteen embryos were recovered nonsurgically at day 6.5 after induced ovulation from Welsh pony mares and were evaluated for cellular changes that occur because of exposure to the cryoprotectant with or without the freeze and thaw process. Day 6.5 horse embryos were either (i) frozen and thawed using glycerol as cryoprotectant ($n = 6$), (ii) given only the glycerol treatment ($n = 5$), or (iii) washed in phosphate-buffered saline (PBS) the same number of times as in the glycerol treatment ($n = 5$). After treatments, embryos were incubated in Minimum Essential Medium (MEM), supplemented with BSA, glutamine, antibiotics and buffered with Hepes, for 1 h for one embryo per group and for 6 h for the others. After histological fixation, embryos were serially sectioned. On observation by light microscopy, the total numbers of interphasic, mitotic and pycnotic nuclei of each embryo were counted. Electron microscopy was used to evaluate the damage to the fine structure of intracellular organelles. The proportion of mitotic cells did not differ among groups (control: 2.3%; glycerol-treated: 1.8%; frozen-thawed: 1.3%). There were significant differences in the proportion of pycnotic cells both between control ($12.8\% \pm 5.6$) and glycerol-treated embryos ($39.4\% \pm 15.9$) ($P < 0.05$) and between control and frozen-thawed embryos ($42.2\% \pm 14.9$) ($P < 0.001$), but no difference was found between treated embryos (glycerol-treated and frozen-thawed embryos). Degenerated cells were not localized in the same place in each embryo and no ultrastructural alteration was uniformly observed among every embryo of each group, but inner cell mass (ICM) cells were affected most by treatments ($P < 0.001$). These results indicate that cryopreservation induces considerable cellular damage. The deleterious effects of glycerol alone appear very important and further research is needed to find a suitable cryoprotectant for the horse embryo.

Introduction

Cryopreservation and export of embryos have become an integral part of the bovine embryo transfer industry: in Europe more than 78 000 frozen bovine embryos were transferred in 1990 (Thibier, 1991). In contrast, freezing of equine embryos is not yet popular in the equine industry because limited success has been reported with this technique. After the first pregnancy which aborted at 2 months of gestation (Griffin *et al.*, 1981), the birth of the first foal produced from a frozen-thawed embryo was reported by Yamamoto *et al.* (1982). Since then several attempts at freezing equine embryos have been reported (Slade *et al.*, 1984; Takeda *et al.*, 1984; Boyle *et al.*, 1985; Czlonkowska *et al.*, 1985; Slade *et al.*, 1985a,b; Wilson *et al.*, 1986; Sirois *et al.*, 1987; Farinasso *et al.*, 1989; Seidel *et al.*, 1989; Squires *et al.*, 1989; Bruyas *et al.*, 1990; Lagneaux and Palmer, 1991; Skidmore

et al., 1991): just over 200 frozen-thawed embryos have been transferred and mean pregnancy rates were lower than 20%. Most of these results were achieved with the same freezing protocols as used for bovine embryos using glycerol as cryoprotectant. Some of these experiments showed better results with small embryos ($\leq 200 \mu\text{m}$).

In two previous studies on the effects of cryopreservation on the ultrastructure (Wilson *et al.*, 1987) and on the metabolic activity (Rieger *et al.*, 1991) of horse embryos, it was suggested that the treatment with the cryoprotectant, glycerol, rather than the freezing-thawing procedure induces most of the damage in cryopreserved horse embryos.

The purpose of the present study was to compare fresh, cryoprotectant-treated and frozen-thawed horse embryos using light and electron microscopy to determine the number and the types of cell that are degenerated and to elucidate the ultrastructural modifications during cryopreservation and subsequent thawing.

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Materials and Methods

Collection of embryos

Oestrus in 33 Welsh pony mares was synchronized with intravaginal sponges containing 500 mg of allyl trenbolone (Regumate: Roussel Uclaf, Paris). Vaginal sponges were withdrawn seven days after insertion and an i.m. injection of 125 µg cloprostenol, a prostaglandin analogue (Estrumate: Pitman Moore, Meaux) was given (Palmer, 1979).

The onset of oestrus was detected by daily teasing with a stallion; follicular growth and ovulation were then checked by daily ultrasound examination. When a growing follicle reached 33 mm or more, 25 mg crude equine pituitary gonadotrophin (CEG) (Duchamp *et al.*, 1987) was injected i.v. Previous observation showed that ovulation occurred at a mean of 36 ± 2 h after injection of CEG in 75% of mares (Bézar *et al.*, 1989).

Artificial inseminations were performed 24 h after injection of CEG with 200×10^6 spermatozoa freshly ejaculated from the same pony stallion and diluted in 10 ml of milk extender (Palmer, 1984).

Nonsurgical embryo collections were performed 8 days after ovulation induction, that is 6.5 days after expected ovulation, by a transcervical flush of the uterus with phosphate-buffered saline (PBS) containing 0.2% BSA at 37°C (IMV, l'Aigle, France) as described by Lagneaux *et al.* (1988).

From the 33 pony mares only 23 collections, in well-synchronized or responding females were performed. Seven mares with one ovulation and one with double ovulations did not produce an embryo. Fourteen mares with one ovulation had produced one embryo and one with double ovulations had produced two embryos.

The embryos were recovered from the flush medium, washed ten times in PBS medium containing 0.4% BSA (F1) (IMV, l'Aigle) at 22°C, under a laminar flow hood. The morphology of the embryos was evaluated with an inverted microscope, and their diameter (including the zona pellucida) measured using a micrometer eyepiece. All embryos were judged morphologically normal: quality score 1 or 2 in the classification system used by McKinnon and Squires (1988). All 16 embryos were in development stage from early to mid-blastocyst, ranging in size from 167 to 267 µm (mean 196.8 µm). In all embryos, a capsule was observed between the zona pellucida and trophoblast cells.

Experimental treatments

The 16 embryos were assigned to three treatment groups in a stratified random fashion. The diameter of embryos respectively of 186.6 ± 17.5 (controls), 204.6 ± 31.9 (glycerol treated) and 198.9 ± 36.5 µm (frozen-thawed) did not differ among the three groups indicating that the three groups were morphologically equivalent before treatment.

Frozen-thawed embryos (group F, $n = 6$) were successively equilibrated at 22°C for 5 min in each of 2.5%, 5.0%, 7.5% and 10% v/v of double-distilled glycerol (Prolabo, Paris, France) in F1 (Czlonkowska *et al.*, 1985), and taken up in 50 µl in 0.25 ml freezing straws (IMV, l'Aigle) (Lagneaux *et al.*, 1988). The straws were cooled from room temperature to -7°C at approximately $-3^\circ\text{C min}^{-1}$ by placing them in an alcohol bath freezer precooled to -7°C . They were seeded and held for

5 min, then cooled at $-0.3^\circ\text{C min}^{-1}$ to -30°C and plunged and stored in liquid nitrogen (Wilson *et al.*, 1986). After 3–5 days, the straws were thawed in water at 37°C (60 s). The embryos were recovered from the straws and placed in fresh 10% glycerol in F1 for 5 min at 22°C. The cryoprotectant was diluted by moving the embryo through six successive baths of decreasing glycerol concentration: 8.3%, 6.7%, 5.0%, 3.3%, 1.6% and 0% in F1. An equilibration time of 5 min was allowed at each step (Slade *et al.*, 1985b). One embryo from this group was lost during the freezing procedure.

Glycerol-treated embryos (group G, $n = 5$) were passed through the same increasing concentrations of glycerol and then immediately moved through the decreasing concentrations of glycerol at 22°C without freezing and thawing.

Control embryos (group C, $n = 5$) were passed through 11 washes with F1 (same number of washes as the treated embryos) for 5 min at each step at 22°C.

After their respective experimental treatments, four of the five embryos of each group were cultured for 6 h at 37°C in four-well plates in 1.5 ml essential medium with Hank's salts (MEM) (Serva, Heidelberg) supplemented with 0.4% w/v essential fatty acid-free BSA (Sigma, St Quentin Fallavier), 2 mmol glutamine l^{-1} (Merck, Dormstadt), 100 iu penicillin ml^{-1} and 100 µg streptomycin ml^{-1} (Gibco, Grand Island) and buffered with 20 mmol Hepes l^{-1} (Serva, Heidelberg). The fifth embryo of each group was cultured for only 1 h in the same conditions.

Histological procedures

After their culture, the embryos were examined with an inverted microscope and their diameters were measured. Immediately after, they were fixed at 4°C in 2% glutaraldehyde in 0.175 mol cacodylate buffer l^{-1} at pH 7.3, post fixed in 2% osmium tetroxide, dehydrated in a graded ethanol series and embedded in Epon 812.

All embryos were serially sectioned into semithin sections (1 µm thick). Every fifth section was stained with 0.5% hot toluidine blue on glass slides for light microscopy. From each of the 15 semithin sections, five ultrathin sections (0.07 µm) were cut with a diamond knife. Ultrathin sections were contrasted with uranyl acetate and lead citrate before electron microscope examination.

The nuclear state (normal or pycnotic, interphase or mitotic) of all embryonic cells of each embryo was assessed on the semithin sections. The number and the size of each class of nucleus were determined by microscope examination using a computer analysis system (Tandom VIDS IV manual, BMS, Cambridge). The total number of cells in each embryo was estimated from the observed number of nuclei: pycnotic nuclei were smaller than 5 µm (3.85 ± 0.35 µm) (only every fifth section was examined) and the counted number was not corrected; in contrast, interphase nuclei were greater than 5 µm (8.73 ± 2.32 µm) and the observed number was corrected by the multiplying coefficient c proposed by Amann (1970):

$$c = e/(e + ((Dm. 0.5)^2 - (Dm. 0.5)^4)^{0.5}) \text{ with } e = 5 \mu\text{m and } Dm = \text{mean diameter of nucleus.}$$

The mitotic cells were easily located on each section and they were counted only once.

Table 1. Gross morphological evaluation of horse embryos before treatment and after culture

Treatment groups (number of embryos)	Initial diameter (mean $\pm$ SD) (μ m)	Increase in diameter (mean $\pm$ SD) (%)	Quality score		Number of embryos after culture with			
			Before treatment (mean $\pm$ SD)	After treatment (mean $\pm$ SD)	Extruded blastomeres	Blastocoel collapse	Crumpled capsule	Darkened cells
Control (C) (n = 5)	186.6 $\pm$ 17.49	8.72 $\pm$ 4.82	1.2 $\pm$ 0.45	1.8 $\pm$ 0.45	3/5	1/5	0/5	0/5
Glycerol (G) (n = 5)	204.6 $\pm$ 31.88	3.86 $\pm$ 5.02	1.2 $\pm$ 0.45*	3.2 $\pm$ 0.84*	5/5 (1) ^a	4/5 (1) ^a	3/5	1/5
Frozen–thawed (F) (n = 5)	198.9 $\pm$ 36.50	5.2 $\pm$ 5.66	1**	2.8 $\pm$ 0.84**	5/5	3/5 (2) ^a	2/5	5/5

^aWithin parentheses number of embryos showing very important signs of alteration.

*Values with the same superscript in the same line are significantly different ($P < 0.02$).

**Values with the same superscript in the same line are significantly different ($P < 0.01$).

Statistical analysis

Intergroup analysis comparisons were made using the Mann–Whitney U-test, and least-square regression lines and Pearson product–moment correlation coefficients were calculated by standard methods (Schwartz, 1969). Differences in quality score of morphological assessment before and after treatment and culture and differences between numbers of inner cell mass (ICM) and trophoblast cells of embryos were tested with a paired Student's *t* test (Schwartz, 1969).

Results

After treatment and culture, there was no notable change in the morphology of the control embryos, and they exhibited a mean increase in diameter of $8.72 \pm 4.82\%$ (Table 1). However, most embryos in the other two groups showed some damage: compact blastomeres and blastocoel collapse resulting in shrinkage with cellular separation from the capsule, crumpled capsule, extruded and isolated blastomeres in the perivitellin space and darkened cells.

After treatment and culture, only one glycerol-treated (246B) and two frozen–thawed embryos were still assigned a quality score of 2, but the other 7 in groups F and G were evaluated as grade 3 or 4 (Table 1). The increases of quality score between before and after treatment and culture were statistically significant in group G ($P < 0.02$) and group F ($P < 0.01$). There was also a significant difference ($P < 0.05$) in the variation of quality score before and after treatment and culture between the control group (C) and the treated groups (G + F). Treated embryos showed a mean increase in diameter of only $3.86 \pm 5.02\%$ (group G) and $5.2 \pm 5.66\%$ (group F). The rates of increase in diameter did not differ among the three groups.

Some of the first semithin sections of one embryo and most sections of another embryo could not be observed. For the 13 other embryos every fifth section was examined. There were between 24 and 38 sections per embryo depending on its size.

The mean estimated number of cells of all embryos ($\pm$ SD) was 624 ± 184 . This number was highly positively correlated

with embryo volume ($r = 0.81$, $P < 0.001$). Number of cells did not differ among the three treatment groups (Table 2). The proportion of mitotic cells was not significantly different among groups (control: 2.3%; glycerol-treated: 1.5%; frozen–thawed: 1.3%). There were significant differences in the proportion of pycnotic cells both between control (group C: $12.8\% \pm 5.6$) and glycerol-treated (group G: $39.4\% \pm 15.9$) embryos ($P < 0.05$) and between control (group C) and frozen–thawed (group F: $42.2\% \pm 14.9$) embryos ($P < 0.01$). There was no significant difference between treated embryos (groups G and F).

Light and electron microscope observations showed that these horse early blastocysts had a centrally located inner cell mass (ICM) (Fig. 1a). The proportion of ICM cells was $32.1 \pm 2.1\%$ in the control, early blastocysts. The expanding blastocoel was clearly visible as irregular cavities between the inner cells (Fig. 1a; Fig. 2a). These cells of ICM had protoplasmic processes of different forms which extend from one cell to another (Fig. 2a, b). The trophoblast cells formed a single layer adjacent to the capsule and were connected by junctional complexes (Fig. 2c) and their outer surfaces were covered with numerous microvilli (Fig. 2d). The two acellular layers, the zona pellucida and the horse specific capsule, could be clearly recognized (Fig. 1a; Fig. 2d). Some embryos showed degenerating cellular material wedged in between the zona pellucida and the capsule (Fig. 1b; Fig. 2d). A copious lipid stock was stored in any size droplets (some were very large) within most of the cells (Fig. 1c; Fig. 3a) and even free in the blastocoel. The cytoplasm had a dark appearance because of its wealth of subcellular components: rough endoplasmic reticulum and free ribosomes (Fig. 3a), and Golgi lamellae (Fig. 3b) were particularly abundant. Many vesicles, some of these were very large, were observed particularly within trophoblast cells in the peripheral area of cytoplasm (Fig. 1a, d; Fig. 2d). The mitochondria were also very numerous. They were small and cylindrical and had a dense appearance in cells of two control embryos (Fig. 3b). However, for the three other control embryos, most mitochondria were very clear, spherical and markedly swollen (Fig. 3c).

The morphological aspect of two embryos of each treated group (G: 237, 242; F: 281, 227) were very different. They were

Table 2. Results of histological analysis after culture of horse embryos

Treatment groups (number of embryos)	Total number of cells	Mitotic cells (%)	Pycnotic nuclei (%)	Inner cell mass		Trophoblastic cells	
				Number of cells	Pycnotic nuclei (%)	Number of cells	Pycnotic nuclei (%)
Control (C)							
220 ^a	408	3.2	6	117	8	291	5
246A	479	3.3	10	157	18	322	12
173	513	2.5	15	163	23	350	11
202	746	1.1	12	251	31	495	2
283	770	1.3	21	259	29	511	17
Mean	583.2	2.3	12.8* [‡]	189.4	21.8 ^{†*}	393.8	9.4*
SD	164.2	1.0	5.6	62.5	9.3	102.0	3.9
Glycerol (G)							
225 ^a	962	2	20	289	46	673	9
264	397	3.3	35	187	57	210	15
237 ^b	(116)	0	53	CCA	CCA	CCA	CCA
246B	529	1.9	33	192	49	337	24
242	708	0.3	56	CCA	CCA	CCA	CCA
Mean	649.0	1.5	39.4*	222.7	50.7*	406.7	16.0
SD	244.5	1.4	15.9	57.5	5.7	239.2	7.5
Frozen-thawed (F)							
274 ^{ab}	(284)	1.4	32	(131)	45	(153)	20
214	429	1.6	42	127	75	302	29
227	563	1.1	33	CCA	CCA	CCA	CCA
278	763	1.7	36	236	59	527	26
281	843	0.9	68	CCA	CCA	CCA	CCA
Mean	649.5	1.3	42.2 [‡]	181.5	59.3 [†]	414.5	25*
SD	188.3	0.3	14.9	77.1	14.5	159.1	4.6

^aEmbryos cultured for only one hour after treatment.

^bSome sections of those embryos could not be observed. Values in parentheses were not included in means.

† or *Values with the same superscript in the same column are significantly different ($P < 0.05$).

‡Values with the same superscript in the same column are significantly different ($P < 0.01$).

CCA: central compact aggregate.

composed of a large central compact cell mass in which the majority of nuclei were normal. No blastocoel was observed. This central cellular aggregate (CCA) was an association of trophoblastic and ICM cells and it was impossible to differentiate them for a separate identification by the microscope examination. Some cells with pycnotic nuclei were in this CCA but most degenerated cells (60–85%) were isolated in the large space between this mass and the capsule (Fig. 1b). Before fixation, the inverted microscope evaluation of these four embryos had already shown that they were very shrunken with extruded and isolated cells in the perivitelline space. These four embryos with one exception had the highest proportions of pycnotic nuclei and degenerated cells (Table 2).

The three other glycerol-treated and the three other frozen-thawed embryos had the same histological and cytological organization as control embryos with a central ICM and peripheral trophoblast cells. Nevertheless degenerated cells were located mainly in the ICM, some of them were free in the blastocoel and a few peripheral cells were damaged (Fig. 1e, f). If all dead cells free in the blastocoel were ICM cells, only 50%

or less of ICM cells would be alive. In this condition, there was a significant difference ($P < 0.001$) in the proportion of pycnotic nuclei between ICM ($55.0 \pm 10.9\%$) and trophoblast ($20.5 \pm 7.4\%$) of the six treated embryos. There were also significant differences in the proportion of pycnotic cells in ICM between control and glycerol-treated embryos ($P < 0.05$) and both in ICM and in trophoblast between control and frozen-thawed embryos ($P < 0.05$).

Junctions and cytoplasmic organelles of the non-degenerated cells of all glycerol-treated and frozen-thawed embryos were the same as those observed in the control embryos. The same spherical and very swollen mitochondria as those observed in some control embryos were also seen in two glycerol-treated and three frozen-thawed embryos (Fig. 3d).

Discussion

The total number of embryonic cells counted here shows that early horse blastocysts are composed of more cells than those

Fig. 1. (a) A light micrograph of a control embryo: an early blastocyst with a capsule (C) and a zona pellucida (ZP). Blastocoel is clearly visible as irregular cavities (star) inside the centrally located inner cell mass (ICM). Trophoblast cells (TC) have a peripheral location. (b) A glycerol-treated embryo. There is a compaction of blastomeres and blastocoel collapse resulting in shrinkage. All living cells are aggregated in a central location, many pycnotic cells (P) are excluded and isolated between the centrally compacted cell aggregate (CCA) and the crumpled capsule (C). Note the degenerating cellular material (DC) wedged in between the capsule (C) and the zona pellucida (ZP). (c) A few cells of a frozen-thawed embryo with different nuclear states: interphase (I), mitosis (M), pycnosis (P). There are many lipid droplets (L). (d) A few trophoblast cells of a glycerol-treated embryo near the capsule (C) with numerous large vacuoles (V) in the peripheral area of cytoplasm. (e) A frozen-thawed embryo with pycnotic cells (P) in the inner cell mass (ICM). (f) A frozen-thawed embryo with pycnotic cells (P) free in blastocoel. Scale bar = 25 µm.

of other domestic species. Early bovine blastocysts are composed of about 60–80 cells, the expanded blastocysts of 90–250 cells, the hatched blastocysts of 150–400 cells and the day 10 cow embryos of 500–700 cells (Renard, 1985; Sripongpun, 1986; Dorland *et al.*, 1988; Nibart *et al.*, 1988; Marquant-Le-Guienne *et al.*, 1989; Iwasaki and Nakahara, 1990). There

would be about 300 cells in the sheep day 8 expanded blastocysts (Wintenberger-Torres and Flechon, 1974) and 80–100 blastomeres in the pig day 5 embryos (Renard, 1985). Nevertheless, our results are in agreement with the results from studies of horse embryos by Slade *et al.* (1985b) and by Clark *et al.* (1987).

Fig. 2. (a) Electronmicrograph of ICM cells of a control, early blastocyst: note irregular cavities of expanding blastocoel (BI) between the cells that have protoplasmic processes. There are some lipid droplets (L). (b) ICM cells of a control embryo: junction (arrow) between cells. (c) Trophoblast cells of a control embryo. Note the junctional complexes between cells (arrow) and the microvilli (m) on the outer surface near the capsule (C). (d) Trophoblast cells of a control embryo. There are many microvilli (m) on the outer surface. Note a degenerating cellular material (arrowhead) wedged between the zona pellucida (ZP) and the capsule (C). Scale bar = 1 μ m.

In the study reported here more cells were found than in our previous report (Rieger *et al.*, 1991), but in this earlier study, the classic histological analysis gave less precise information than the serial fine process used here.

The presence of more blastomeres in horse embryos than in embryos of other species may be due to the fact that early blastocyst stage is reached later after ovulation in equine

embryos than in ruminant embryos or to a faster cell cycle in horse embryos than in ruminant embryos.

However, the proportion of mitotic cells in the control embryos is not very high because only metaphase cells were counted as mitosis and metaphase is a short period during the cell cycle. This mitotic rate agreed with our first study (Rieger *et al.*, 1991) and with those reported for embryos of other species

Fig. 3. Electronmicrograph of control and treated embryos (a) ICM cells of a control embryo with an abundant rough endoplasmic reticulum (arrow), free ribosomes (star) and large lipid droplets (L). (b) ICM cells of a control embryo with many vesicles (v), Golgi apparatus (arrowhead) and small cylindrical dense mitochondria (M). (c) Trophoblast cells of a control embryo with very clear, spherical and markedly swollen mitochondria (M). (d) Trophoblast cells of a glycerol-treated embryo with the same clear, spherical and markedly swollen mitochondria (M) as embryo in (c). Note Golgi apparatus (arrowhead). Scale bar = 1 μ m.

(Renard, 1985; Dorland *et al.*, 1988; Marquant-Le-Guienne *et al.*, 1989; Nibart *et al.*, 1989).

Our observations on the structure and ultrastructure of early horse blastocysts confirm studies of Flood *et al.* (1982) and Wilson *et al.* (1987). Nevertheless the proportion of ICM cells of horse early blastocysts has not been estimated in other studies. The proportion evaluated in this study is greater than that reported by Iwasaki *et al.* (1990) in bovine blastocysts but

in accordance with those described in bovine blastocysts by Renard (1985), Sripongpun (1986) and Marquant-Le-Guienne *et al.* (1989).

The purpose of our ultrastructural investigations was to test for early impairment of subcellular components owing to glycerol treatment or freezing or both processes. Stages of cell injury have been described by Trum *et al.* (1980). Dilatation of the endoplasmic reticulum or condensation of mitochondria, which

are first symptoms of cell injuries, were not seen in the study reported here. We have previously found modifications in metabolic activity of glycerol-treated and frozen-thawed embryos that would suggest a loss of the functional integrity of the mitochondria. Wilson *et al.* (1987) showed that cryopreservation or, to a lesser extent, glycerol treatment results in changes of the mitochondria of horse embryos, including thickening of the cristae, coalescence of the inner membrane and blebbing of the outer membrane. These alterations were not seen in the study reported here. The modification of the staining contrast of the lipid in the embryos that had been exposed only to the cryoprotectant by Wilson *et al.* (1987) was not observed in the study reported here. Half of the embryos of the three groups displayed a massive swelling of mitochondria which would be regarded as early symptoms of cellular necrosis, as Trum *et al.* (1976) showed in dog myocardial cells after total ischemia. In the same way, Malinin (1973) reported that rhesus kidney dimethyl sulfoxide (DMSO)-treated cells display a swelling of mitochondria and damage to their membranes and cristae. Nevertheless, in the present study it does not seem to be the glycerol or freezing or a combination of these processes that induced these ultrastructural modifications, because some control embryos showed the same type of mitochondria. The onset of cellular necrosis in control embryos would be induced by culture conditions after treatment. Renard (1985) showed that culture during a few hours induces some damage to cow and pig embryos, and Clark *et al.* (1987) reported that culture of horse embryos in minimum essential medium during 12–24 h with Hank's salts reduced their viability. Pruit *et al.* (1991) saw similar modifications of mitochondria (round with few parallel cristae) in remaining viable cells of non-viable horse embryos after culture during 24 h. In the same way, none of the three embryos of the present work that were cultured for only 1 h displayed swollen mitochondria.

The proportion of pycnotic cells of control embryos is lower than that previously observed in control embryos cultured for 12 h (Rieger *et al.*, 1991), but higher than that in fresh embryos immediately fixed after recovery ($5.8\% \pm 3.4$) (J-F. Bruyas, unpublished). We had chosen to culture embryos for 6 h after treatment to evaluate the cellular damage more easily. Indeed, during this time of culture the injured cells would either restore their integrity or degenerate and give a pycnotic nucleus which was counted in the histological study. If embryos were fixed immediately after treatment, injured cells would not display pycnotic nuclei since a certain time is required to achieve this stage of necrosis. Nevertheless, the glycerol-treated and the frozen-thawed embryos which were cultured for only 1 h have a higher proportion of pycnotic cells than every other control embryo. Wilson *et al.* (1987) made the same observation in one frozen-thawed embryo that had been cultured for 1 h after thawing. One hour of culture is perhaps sufficient time to observe the necrosis of embryonic cells after injuries of cryopreservation.

In histological studies on embryos of other species (Renard, 1985; Dorland *et al.*, 1988; Marquant-Le-Guienne *et al.*, 1989; Nibart *et al.*, 1989), the proportions of pycnotic cells of fresh embryos or embryos cultured for a short time are similar to those observed in the horse control embryos in this study. We have already reported (Rieger *et al.*, 1991) that glycerol-treated and glycerol-thawed embryos show statistically significant higher proportions of degenerated cells than do control embryos.

These rates are comparable to those estimated by Slade *et al.* (1985b) in frozen horse embryos, but higher than those reported for frozen bovine blastocysts (Camous *et al.*, 1984; Renard, 1985) and for frozen mouse blastocysts (Low *et al.*, 1986).

Wilson *et al.* (1987) and Barry *et al.* (1989) showed in horse embryos that inner cells were more damaged than peripheral cells during the cryopreservation procedure. In contrast, Renard (1985) found as many degenerated cells in trophoblast as in ICM of bovine frozen-thawed and cultured blastocysts. In the present work, some embryos of each treated group (G and F) showed more damage in the ICM. In the other embryos, viable cells were grouped in a central compact mass and degenerated blastomeres were dispersed in peripheral space. Pycnosis which occurs after 6 h of culture in non-treated embryos is less severe and spread in whole embryos, but ICM cells seem to be more affected.

This study confirms that the detrimental effects of treatments such as glycerol freezing and culture seem to be exerted particularly on the ICM cells which are more sensitive to adverse conditions. Similar findings have been reported following the culture of horse (Pruit *et al.*, 1991) and bovine embryos (Camous *et al.*, 1984; Sripongpun, 1986; Nibart *et al.*, 1988; Marquant-Le-Guienne *et al.*, 1989).

In this work as in the previous study (Rieger *et al.*, 1991) there was no difference between rates of pycnosis for glycerol-treated and frozen-thawed embryos. This latest result agrees with the previous suggestion of Wilson *et al.* (1987), Lagneaux and Palmer (1991) and Rieger *et al.* (1991) that the poor results of freezing of horse embryos is, in part, due to damage induced by the treatment with the cryoprotectant glycerol. This deleterious effect of glycerol *per se* justifies attempts to use other cryoprotectants (Seidel *et al.*, 1989).

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