Deep freezing of horse embryos*

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Summary. Fourteen horse embryos recovered non-surgically on Days 6–8 after ovulation (Day 0) were cooled slowly to -35°C (7 embryos) or -40°C (7 embryos) and stored in liquid nitrogen (-196°C) for 4–98 days. Surgical transfer of the thawed embryos to unmated recipient mares that had ovulated -2 to $+1$ days with respect to the embryo donors resulted initially in the establishment of 4 conceptuses. However, only one mare maintained her pregnancy to term.

Introduction

Deep freezing of embryos is now well established in cattle (Wilmot & Rowson, 1973; Willadsen, Polge & Rowson, 1978; Lehn-Jensen, 1980; Renard, Ozil & Heyman, 1981; Massip, van der Zwalm, Hanzen & Ectors, 1982; Niemann, Lampeter, Sacher & Krieff, 1982; Leibo, 1983; Renard, Heyman, Leymonie & Plat, 1983), sheep (Bilton & Moore, 1976; Willadsen, Polge, Rowson & Moor, 1976; Willadsen, 1977; Tervit & Goold, 1984) and laboratory rodents (Whittingham, Leibo & Mazur, 1972; Wilmot, 1972; Whittingham & Adams, 1976; Tsunoda, Shimohara, Yzumi, Soma & Sugie, 1979; Landa, 1981). However, there has been less success to date when attempts have been made to freeze pig (Polge, Wilmot & Rowson, 1974) and horse embryos. Yamamoto, Oguri, Tsutsumi & Hachinohe (1982) reported the birth of one live foal following the transfer of 11 embryos recovered and frozen at Day 6 after ovulation; a further 2 of these frozen embryos were thought to have resulted in pregnancies that developed only to Days 32–43. Takeda, Elsdon & Squires (1984) obtained 2 live foals following the surgical transfer of 4 frozen-thawed Day-6 embryos to 3 recipient mares and Slade, Takeda & Squires (1985) reported an experiment in which 9 of 17 recipient mares remained pregnant at Day 50 after surgical transfer of frozen-thawed Day-6 blastocysts.

The present study was undertaken to examine the effects of two methods of freezing on the morphological appearance and viability of horse embryos.

Materials and Methods

Embryo recovery. During May to August, 1983, 14 embryos were recovered non-surgically on Days 6–8 after ovulation (Day 0) from the uteri of Welsh Pony and Welsh Cob mares that had been mated naturally to one of 2 Welsh Pony stallions during the previous oestrus. Ovulation in the donor and recipient mares was diagnosed by a combination of rectal palpation of the ovaries and measurement of progesterone concentrations in peripheral plasma samples recovered daily during oestrus and for 3 days thereafter.

The embryo recovery technique described by Allen (1982) was used. A Gibbon balloon catheter

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(20-French gauge; Franklin Medical, High Wycombe, Bucks, U.K.) was inserted through the cervix and the cuff inflated with 40 ml air to occlude the internal os. Three aliquants (400–1200 ml) of flushing medium (Ovum Culture Medium; Flow Laboratories Ltd, Rickmansworth, Herts, U.K.) were sequentially infused into the uterus and recovered by gravity flow into sterile glass measuring cylinders. If the embryo was not visible the cylinders were allowed to stand at room temperature (18–25°C) for 20 min before the bulk of the medium was carefully aspirated from the surface. The remaining 20–30 ml was then searched with the aid of a stereoscopic dissecting microscope.

Embryo freezing. The embryos were held in 15–25 ml enriched Dulbecco's phosphate buffered saline (PB1; Whittingham, 1974) for 30–120 min at 18–25°C before initiating the freezing process. Freezing was carried out using a slight modification of the method described by Willadsen (1980) for sheep and cattle embryos. Glycerol, at a final concentration of 10% (v:v), was used as a cryoprotectant and was added at room temperature in 4 steps (2.5, 5, 7.5 and 10%), each of 10 min. The embryo, suspended in 0.25 ml freezing medium (10% glycerol in Medium PB1) in a small glass test tube (50 mm long, 6.25 mm o.d., 4.75 mm i.d.; Glass Wholesale Supplies, London, U.K.) was placed in the freezing machine (Model R-202; Planar Products, Sunbury-on-Thames, Berks, U.K.) at 24°C. Initial cooling was at the rate of 1°C/min to –6°C when the glass tubes were seeded with forceps pre-cooled in liquid nitrogen. The cooling rate was then reduced to 0–3°C/min. The first 7 embryos recovered were cooled to –35°C (Group I) while the next 7 were cooled to –40°C (Group II). On reaching these temperatures the tubes were plunged into liquid nitrogen (–196°C) and stored for 4–98 days.

Embryo thawing and transfer. The embryos were thawed rapidly by dropping the freezing tubes into a water bath at 37°C. Glycerol was then removed in four 10-min steps by passing the embryos through a series of Petri dishes containing Medium PB1 with reducing concentrations of glycerol (10, 7.5, 5, 2.5%). Finally, the embryos were rinsed for 10–20 min in Medium PB1 without glycerol before being transferred to recipient mares. One Group-I and 6 Group-II embryos were cultured in Medium PB1 supplemented with 20% (v:v) fetal calf serum (FCS; Gibco Laboratories Ltd, Uxbridge, Middlesex, U.K.) at 37°C in an atmosphere of 5% CO₂:95% air for 24–72 h before being transferred. Six of the embryos showed poor or doubtful morphology immediately after thawing and they were cultured in an attempt to be more certain of their viability before embarking upon the trouble and expense of surgical transfer to recipient mares. For the remaining embryo a recipient mare was not available at the required time.

Embryos were transferred surgically as described by Allen (1982) into recipient mares that had ovulated from 1 day ahead (+1) to 2 days behind (–2), relative to respective donor mares. Pregnancy was diagnosed from Day 16 by realtime ultrasound scanning of the uterus (Simpson *et al.*, 1982) and monitored by radioimmunoassay of peripheral plasma progesterone concentrations (Newcomb, Booth & Rowson, 1977) and haemagglutination-inhibition assay of serum chorionic gonadotrophin (horse CG) concentrations (Allen, 1969).

Results

The stages of development of the embryos collected, and the effects of freezing and thawing, are summarized in Table 1. Immediately after thawing most of the embryos were very shrunken and collapsed. Four of the 7 Group-I (–35°C) embryos assumed a normal appearance during removal of the glycerol whereas only 2 of the 7 Group-II (–40°C) embryos became fully expanded after removal of the cryoprotectant. Moreover, structural damage to the zona pellucida was observed more frequently in the embryos of Group II than of Group I (6/7 and 3/7 respectively). Three of these Group-II embryos were at the large expanded blastocyst (LEB) stage when frozen and all 3 showed considerable morphological damage after thawing.

Table 1. Recovery, freezing, in-vitro culture and transfer of horse embryos

Donor mare	Days of embryo collection (ovulation = Day 0)	Stage of embryo*	Time in liquid N ₂ (days)	Duration in culture after thawing (h)†	Synchrony between donor and recipient mares (± days)	Result‡
<i>Group I</i>						
TM	6-5	SEB	10	NC	-1	NP
HT	7	EB	98	NC	-2	NP
BG	7	SEB	4	24	-1	NP
DF	7	SEB	19	NC	-2	NP
AC	7	SEB	11	NC	-2	NP
JR	7	UB	5	NC	-2	Pregnant; conceptus resorbed at Days 66-74
AS	7	SEB	9	NC	-2	Pregnant; colt foal born on Day 327
<i>Group II</i>						
HM	6	UB	23	24	+1	TV; resorbed Days 32-40
HM	7	SEB	12	NC	-1	NP
BG	7	EB	6	48	-2	NP
MQ	7	M	15	24	+1	NP
MN	7	LEB	8	24	0	NP
DB	8	LEB	28	24	0	NP
AC	8	LEB	14	72	0	TV; resorbed Days 28-36

* M = morula; UB = unexpanded blastocyst; SEB = small expanded blastocyst; EB = expanded blastocyst; LEB = large expanded blastocyst.

† NC = not cultured.

‡ NP = not pregnant; TV = trophoblastic vesicle.

During culture *in vitro* all the Group I and 2 of 6 Group II embryos expanded fully to assume the appearance of normal blastocysts. Three other Group-II embryos that appeared damaged showed partial expansion and the remaining Group-II embryo remained static as a morula (Table 1).

All the embryos were transferred surgically to recipient mares that had ovulated + 1 to - 2 days with respect to the embryo donor. Two Group-I embryos resulted in established pregnancies although only one of these progressed to term with the birth of a live colt foal on Day 327. The other pregnancy failed between Days 66 and 74 despite a normal echogenic appearance of the fetus on Day 66. Normal profiles of horse CG and progesterone were measured in the peripheral circulation of this mare up to the time of pregnancy failure (see Allen, 1975). Two Group-II embryos gave rise to trophoblastic vesicles that showed normal echogenic development between Days 16 and 23. However, an embryo failed to appear in both vesicles at the expected time (Day 23-25) and thereafter they regressed steadily to disappear finally between Days 36 and 40.

Discussion

Although the pregnancy rate obtained in the present study was low, the results nevertheless indicated a number of features that may be important for obtaining higher rates in the future.

First, a definite improvement in the morphology of the embryos after thawing and the pregnancy rates after transfer was observed for Day-7 embryos (blastocysts or small expanded blastocysts) compared with Day-8 embryos (large expanded blastocysts). This is in accord with the conclusion of Slade *et al.* (1985) that early equine blastocysts tolerate cryopreservation better than larger blastocysts. Niemann *et al.* (1982) made a similar observation for cow embryos.

Second, although culturing embryos *in vitro* after thawing helped to evaluate their quality by morphological appearance, it did not improve their viability. None of the 7 cultured embryos resulted in a viable fetus after transfer and the fact that 2 of them gave rise to trophoblastic vesicles suggests the possibility of damage to the inner cell mass during the culture period. Renard *et al.* (1981) noted an improvement in pregnancy rate after transfer of frozen-thawed, cultured Day-8 cow blastocysts. However, the overall survival rates were too low to enable any firm conclusions to be reached and, moreover, the same authors had previously shown that culturing cattle blastocysts for 24 h decreased their viability after transfer (Renard, Menezo, Saumande & Heyman, 1978). We suggest, therefore, that embryo quality should be evaluated immediately after thawing so that morphologically normal embryos can be transferred immediately to recipient mares without further incubation *in vitro*.

The third observation concerns the degree of synchrony between the times of ovulation in the donor and recipient mares. In the experiment involving the non-surgical transfer of embryos between mares, Oguri & Tsutsumi (1982) obtained the highest pregnancy rates when transferring embryos to recipients that had ovulated 1-3 days after the respective donors; pregnancy rate declined sharply when transferring the embryos to recipients that had ovulated before the donor. This relationship appeared to be sustained in the present study in which both established pregnancies resulted from embryos transferred to recipients that had ovulated 2 days after the donors. It seems likely that a degree of negative asynchrony is necessary to compensate for a temporary slowing of the rate of development of the embryo, induced by either or both the transfer and freezing procedures.

The appearance of the embryos after thawing in this study failed to provide a clear indication of the optimum temperature to which equine embryos should be cooled slowly before plunging them into liquid nitrogen. On the whole, the morphology and general appearance of embryos in Group I were slightly better than those in Group II. This observation, together with the fact that both the established pregnancies came from Group-I embryos, would suggest that - 35°C may be preferable to - 40°C. Several Group-II embryos looked quite normal after in-vitro culture, although

they failed to form viable conceptuses after transfer. This may have been related to the previously mentioned adverse effects of culture, including ultrastructural changes that were not detectable at the light microscopic level. Similar problems of permeability may be involved in the reduced ability of large horse blastocysts to survive freezing and thawing, as suggested for cow blastocysts (Massip & Mulnar, 1980).

More fundamental studies are obviously needed to establish optimum methods for freezing and thawing horse blastocysts. None the less, the results of this preliminary experiment, and those of Yamamoto *et al.* (1982) and Takeda *et al.* (1984), have shown that, despite a low rate of success, it is possible to obtain normal foals after transfer of frozen-thawed blastocytes. Cryopreservation of embryos has considerable potential for the breeding of domestic and captive wild equids.

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