SURVIVAL OF RAT EMBRYOS AFTER FREEZING AND THAWING

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(Received 13th December 1974)

Survival of mammalian embryos after storage at sub-zero temperatures was first achieved in the mouse in which all the preimplantation stages were shown to survive storage at -196°C when suitably cooled and thawed (Whittingham, Leibo & Mazur, 1972; Wilmut, 1972; Whittingham, 1974a). Similar procedures have demonstrated that blastocysts of cattle (Wilmut & Rowson, 1973), morulae and blastocysts of sheep (Willadsen, Polge, Rowson & Moor, 1974) and four-cell, eight-cell and morulae of rabbits (Bank & Maurer, 1973; Whittingham & Adams, 1974) will survive storage at -196°C . This communication reports the survival of two-cell, four-cell and eight-cell rat embryos after storage at -196°C for periods up to 3 months.

Superovulation of immature female rats, 26 to 28 days of age (CFHB random-bred strain, Carworth-Europe), with intraperitoneal injections of 25 i.u. PMSG and 10 i.u. HCG given 44 to 48 hr apart produced large numbers of unfertilized oocytes or embryos retarded in development following successful mating which was detected by the presence of spermatozoa or a coital plug in the vagina. As a result of this finding, rat embryos were obtained from naturally mated mature females, 6 to 12 weeks of age, of the CFHB random-bred strain (Carworth-Europe). The day on which spermatozoa or a coital plug was found in the vagina was taken as Day 1 of pregnancy. Two-cell and four-cell embryos were flushed from the oviducts on the morning of Day 2 and Day 3, respectively, and eight-cell embryos were flushed from the oviduct and anterior portion of the uterine horn on Day 4 of pregnancy. Between ten and eighteen embryos were collected from each female. The embryos were collected in a standard culture medium for mouse embryos containing 94.59 mM-NaCl, 4.78 mM-KCl, 1.71 mM- CaCl_2 , 1.19 mM- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.19 mM- KH_2PO_4 , 25.07 mM- NaHCO_3 , 23.28 mM-Na lactate, 0.33 mM-Na pyruvate, 5.56 mM-glucose, 4 mg bovine serum albumin/ml, 100 Units penicillin G (potassium salt)/ml and 50 μg streptomycin sulphate/ml (Whittingham, 1971). They were washed in two changes of medium and transferred to 0.1 ml phosphate-buffered medium (PB1: Whittingham & Wales, 1969) contained in a glass culture tube (12 $\times$ 75 mm). The embryos were equilibrated with 1.5 M-dimethylsulphoxide (DMSO) at 0°C for 15 min by the addition of a further 0.1 ml PB1 containing 3 M-DMSO. The procedures for freezing and thawing were similar to those used for mouse embryos and have been described elsewhere (Whittingham *et al.*,

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1972; Whittingham, 1974b). Various rates of cooling and thawing were studied. Upon thawing, the DMSO was diluted out in a step-wise manner with the culture medium for mouse embryos containing 10% fetal calf serum.

An initial assessment of survival was made by tabulating the proportion of embryos which appeared morphologically normal at recovery since, until rat embryos reach the late eight-cell stage, they do not develop continuously *in vitro* to the blastocyst stage (Mayer & Fritz, 1974; Whittingham, 1975). In the mouse, there is a very close approximation between the number of embryos morphologically normal at recovery and the number that continue development to the blastocyst stage *in vitro* (Whittingham *et al.*, 1972). The proportions of two-cell, four-cell and eight-cell rat embryos which appeared normal at recovery after cooling at various rates from -5°C to -80°C before transfer to liquid nitrogen at -196°C are summarized in Table 1. The range of effective

Table 1. Percentage survival of frozen-thawed rat embryos cooled in 1.5 M-DMSO at various rates to -80°C , stored at -196°C and thawed at approximately $5^{\circ}\text{C}/\text{min}$

Stage of embryo	Cooling rate ($^{\circ}\text{C}/\text{min}$)			
	~ 0.3	~ 0.6	0.8 to 1.0	~ 6.0
Two-cell	57* (5, 58)†	71 (3, 35)	84 (5, 51)	2 (2, 20)
Four-cell	—	70 (4, 67)	—	—
Eight-cell	65 (5, 96)	68 (3, 32)	73 (2, 22)	0 (1, 20)

* % Survival = number of embryos normal after thawing $\times 100/\text{total number of embryos recovered}$.

† Figures in parentheses represent the number of replicates, and the number of embryos frozen.

cooling rates for survival was similar to that found for the mouse (Whittingham *et al.*, 1972), an optimal rate being between 0.6 and $1.0^{\circ}\text{C}/\text{min}$. There was no difference in survival of embryos warmed at $5^{\circ}\text{C}/\text{min}$ (Table 1) and $24^{\circ}\text{C}/\text{min}$ (results not given), but none survived warming at $450^{\circ}\text{C}/\text{min}$, findings similar to those for the mouse.

After the morphological assessment of survival, the embryos were transferred to drops of medium plus 10% fetal calf serum in plastic Petri dishes with a paraffin oil overlay and were cultured at 37°C in 5% CO_2 in air (Brinster, 1963). The embryos were observed after 24 and 48 hr in culture and the developmental stages reached at 48 hr are summarized in Table 2. A small proportion of two-cell embryos, normal at recovery, cleaved to the four-cell stage (25/84, 30%) after 24 hr *in vitro*, but no further development took place. The four-cell embryos failed to cleave in culture but 61% (38/62) of the eight-cell embryos developed into morulae and blastocysts within 48 hr *in vitro*. These findings are similar to the previous results reported for the culture of unfrozen rat embryos (Folstad, Bennett & Dorfman, 1969; Mayer & Fritz, 1974; Toyoda & Chang,

1974; Whittingham, 1975). Thus, even within the limits of assessing viability by continued development *in vitro*, there is a good indication that rat embryos survive storage at -196°C when suitably cooled and thawed over a range of rates optimal for the preservation of mouse embryos.

Table 2. Development of frozen-thawed rat embryos *in vitro*

Stage of embryo frozen	No. recovered/ no. frozen	No. normal (%)	Duration of culture (hr)	Developmental stage at end of culture			
				Four-cell	Eight-cell	Morula	Blastocyst
Two-cell	121/144	84 (69)	48	25	—	—	—
Four-cell	55/67	37 (67)	48	37	—	—	—
Eight-cell*	92/128	62 (65)	48	—	24†	7	31

* Only embryos cooled at ~ 0.3 and $\sim 0.6^{\circ}\text{C}/\text{min}$ (see Table 1) were cultured.

† Seventeen degenerated within the first 24 hr.

The final test of viability of the frozen-thawed rat embryos was made by transfer of the embryos immediately upon thawing to the oviducts of pseudo-pregnant recipients. Pseudopregnancy was induced in mature female rats by mechanical stimulation of the cervix in the evening of the day of pro-oestrus. Nineteen two-cell, twelve four-cell and thirty-three eight-cell embryos considered to be normal at recovery were transferred to the oviducts (three to six embryos/oviduct) of females on the afternoon following induction of pseudopregnancy. The females were examined on Day 18 of pregnancy; two apparently normal live fetuses (11%) resulted from the transferred two-cell embryos, none from the four-cell and three apparently normal live fetuses (9%) from the transferred eight-cell embryos. The over-all viability was low, but no comparison was made of the development of unfrozen rat embryos similarly transferred. The transfer of the eight-cell embryos to the uterus on Day 4 of pseudopregnancy might have increased survival. It is also possible that there was poor induction of pseudopregnancy by the method of cervical stimulation. Nevertheless, the results demonstrate that rat embryos survive storage at -196°C and, although the viability is low compared with that in the mouse, it is anticipated that high rates of survival to term will be obtained after improvement of the methods for embryo culture, embryo transfer and induction of pseudopregnancy.

This work was supported by the Medical Research Council, U.K. I wish to acknowledge the expert technical assistance of Mrs J. Donaldson.

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