THE VIABILITY OF FROZEN-THAWED MOUSE BLASTOCYSTS

D. G. WHITTINGHAM

Physiological Laboratory, Cambridge CB2 3EG

(Received 30th October 1973)

The successful preservation of mouse embryos at sub-zero temperatures has been accomplished with suitably slow rates of cooling and thawing and in the presence of the cryoprotective agent, dimethylsulphoxide (DMSO). In the first report, full-term fetuses and live young were obtained from one-cell, two-cell and eight-cell embryos stored at -196°C for up to 8 days (Whittingham, Leibo & Mazur, 1972) and more recently eight-cell embryos were stored for 8 months at -196°C without any deterioration in viability (Whittingham & Whitten, 1974). The viability of frozen-thawed mouse blastocysts has only been demonstrated by their subsequent expansion during 24 hr in culture. When DMSO was added to the blastocysts at 0°C before freezing and diluted out at 0°C on thawing, the percentage surviving (18%) was low (Whittingham *et al.*, 1972) but higher survival rates (up to 86%) were obtained when DMSO was added and diluted in several increments at 20°C (Wilmot, 1972). This report examines the optimal temperatures for the addition and dilution of DMSO and the survival of frozen-thawed blastocysts to full-term fetuses and live young after transfer to suitably prepared foster mothers.

Blastocysts were flushed from the uterine horns of 6- to 8-week-old super-ovulated mice (CFLP, Carworth-Europe) on the 3rd day after finding the coital plug (the methods used have been described fully elsewhere by Biggers, Whitten & Whittingham, 1971). The blastocysts were collected in a modified Dulbecco's phosphate-buffered medium (PBI) supplemented with 0.33 mM-sodium pyruvate, 5.56 mM-glucose, 3 mg/ml bovine serum albumin and 100 u penicillin G, potassium salt/ml (Whittingham & Wales, 1969). After washing the blastocysts free from debris with two changes of PBI (2 ml/wash), ten to twenty blastocysts were placed in 0.1 ml PBI contained in freezing tubes (10×75 mm). The DMSO in PBI medium was added to samples at 0, 20 and 37°C respectively in two increments of 0.05 ml. All samples were equilibrated for 10 min at the various temperatures before and after each addition of DMSO. The final concentration of DMSO was 1.5 M. Finally, all samples were held at 0°C in an ice-bath for 5 min before transferring them to a seeding bath at -4.5°C . The procedures for freezing and thawing the blastocysts have been described previously (Whittingham *et al.*, 1972). The blastocysts were cooled from 0°C to -80°C at $0.40 \pm 0.07^{\circ}\text{C}/\text{min}$ and transferred immediately to liquid nitrogen (-196°C) for periods ranging between 3 hr and 31 days. No significant differences in survival were observed during these periods of storage at -196°C . The samples were warmed from -110°C to 0°C at $4.25 \pm 0.24^{\circ}\text{C}/\text{min}$. All rates

for cooling and warming were calculated from the time required for the samples to pass from -10 to -70°C or vice versa. Immediately upon thawing, the DMSO was diluted from the samples at 0° , 20° and 37°C in a stepwise manner by the addition of 0.2, 0.2 and 0.4 ml of PBI medium (at the corresponding temperature) at 2-min intervals. The contents of each freezing tube were emptied into an embryological watchglass containing 1 ml medium and the tubes were rinsed with another 1 ml medium to ensure maximum recovery of blastocysts. The blastocysts were recovered, washed in two changes of PBI medium (2 ml/wash) at room temperature, and finally transferred to drops of a modified Krebs-Ringer bicarbonate medium under paraffin oil for culture at 37°C for 17 to 20 hr (Whittingham, 1971). Viability was assessed in the first instance, by the ability of the frozen-thawed blastocysts to expand during culture *in vitro*. The ultimate test of viability was made by transferring the apparently normal expanded blastocysts (three to six blastocysts/horn/female) after 17 to 20 hr in culture to the uterine horns of pseudopregnant females (CFLP, Carworth Europe) mated with vasectomized males (C57BL $\times$ A2G) previously proven sterile; the procedures are described elsewhere (Biggers, Moore & Whittingham, 1965).

Table 1. The effect of adding and diluting DMSO at various temperatures on the survival of frozen-thawed mouse blastocysts

Addition temperature	Percentage of blastocysts showing normal expansion during 17 to 20 hr in culture		
	Dilution temperature		
	0°C	20°C	37°C
0°C	50.7 (6, 73)*	64.4 (6, 73)	75.9 (5, 54)
20°C	40.5 (5, 74)	48.3 (4, 60)	73.3 (4, 39)
37°C	21.3 (6, 75)	58.7 (4, 46)	68.5 (5, 54)

The concentration of DMSO was 1.5 M.

* The number of observations, and the total number of blastocysts appear in parentheses.

The effect of adding and diluting the DMSO at various temperatures on the survival of frozen-thawed mouse blastocysts is summarized in Table 1. The highest survival rates were obtained when the DMSO was diluted from the samples at 37°C irrespective of the temperature at which it was added. The lowest survival was obtained when the DMSO was added at 37°C and diluted at 0°C (21.3%). All other temperature combinations for adding and diluting the DMSO produced intermediate survival rates which suggests that there is an interaction between the temperatures for addition and dilution of DMSO. A more detailed analysis and discussion of these data together with the effects of time for equilibration and concentration of DMSO will appear elsewhere (Whittingham, 1974). Rapid removal of the DMSO from blastocysts at 37°C is clearly essential for good survival, possibly because this may reduce osmotic damage and the toxic effects of the agent (Whittingham & Wales, 1969). The cells of blastocysts exposed to DMSO at 37°C before freezing remain swollen for prolonged periods of time (up to 6 hr) when the DMSO

Table 2. Development of frozen-thawed mouse blastocysts to full-term fetuses and live young after culturing for 17 to 20 hr and transferring to pseudopregnant females

Experiment No.	Time stored at -196°C (days)	No of blastocysts transferred	No. of recipients	No. pregnant	No. transferred to pregnant recipients	No. of fetuses	No. of liveborn	% fetuses and liveborn
1	1	31	5	3	18	7	6	72.2
2	13	104	13	11	88	22	30	59.1
3	31	49	6	3	24	8	7	62.5
Total		184	24	17	130	37	43	61.5

is diluted and at 0°C. Furthermore, the results indicate that, when blastocysts are equilibrated with DMSO at 20°C and 37°C, higher intracellular concentrations of DMSO are probably achieved before freezing which require rapid removal on thawing to minimize any injurious osmotic effects.

Frozen-thawed blastocysts which had expanded during 17 to 20 hr in culture were transferred to pseudopregnant foster mothers. The blastocysts from all treatments were pooled in each experiment and the data are summarized in Table 2. There were no significant differences between the numbers of fetuses and liveborn obtained from blastocysts stored at -196°C for 1 day, 13 days or 31 days. The overall numbers of fetuses and liveborn obtained from the mice which became pregnant (61.5%) compare favourably with previous results (54.1%) for the transfer of frozen eight-cell embryos cultured to blastocysts *in vitro* (Whittingham *et al.*, 1972). All the preimplantation stages of the mouse can be successfully stored at -196°C and their viability on transfer to foster mothers is similar to the transfer results of unfrozen embryos (Mullen & Carter, 1973).

I thank Professor C. R. Austin for his help and encouragement and Mrs J. Donaldson for her expert technical assistance. The work was supported by the Medical Research Council.

REFERENCES

- BIGGERS, J. D., MOORE, B. D. & WHITTINGHAM, D. G. (1965) Development of mouse embryos *in vivo* after cultivation from two-cell ova to blastocysts *in vitro*. *Nature, Lond.* **206**, 732.
- BIGGERS, J. D., WHITTEN, W. K. & WHITTINGHAM, D. G. (1971) The culture of mouse embryos *in vitro*. In *Methods of Mammalian Embryology*, p. 86. Ed. J. C. Daniel, Jr. Freeman, San Francisco.
- MULLEN, R. J. & CARTER, S. C. (1973) Efficiency of transplanting normal, zona-free, and chimeric embryos to one or both uterine horns of inbred and hybrid mice. *Biol. Reprod.* **9**, 111.
- WHITTINGHAM, D. G. (1971) Culture of mouse ova. *J. Reprod. Fert.*, Suppl. **14**, 7.
- WHITTINGHAM, D. G. (1974) Further observations on the freezing and thawing of mouse embryos. *Cryobiology* (in press).
- WHITTINGHAM, D. G., LEIBO, S. P. & MAZUR, P. (1972) Survival of mouse embryos frozen to -196°C and -269°C. *Science, N.Y.* **178**, 411.
- WHITTINGHAM, D. G. & WALES, R. G. (1969) Storage of two-cell mouse embryos *in vitro*. *Aust. J. biol. Sci.* **22**, 1065.
- WHITTINGHAM, D. G. & WHITTEN, W. K. (1974) Long-term storage and aerial transport of frozen mouse embryos. *J. Reprod. Fert.* **36**, 433.
- WILMUT, I. (1972) The effect of cooling rate, warming rate, cryo-protective agent and stage of development on survival of mouse embryos during freezing and thawing. *Life Sciences*, **11**, 1071.