STORAGE OF TWO-CELL MOUSE EMBRYOS *IN VITRO**

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There are reports of the storage at 0–10°C of fertilized rabbit (Chang 1947, 1948; Hafez 1965) and sheep ova (Averill 1956; Kardymowicz *et al.* 1963; Kardymowicz, Kardymowicz, and Grochowalski 1966; Kardymowicz, Kardymowicz, and Kremer 1966). However, the storage of fertilized mouse ova *in vitro* does not appear to have been studied. Wales (1969) found that glycolysis at 37°C by cultured blastocysts was not impaired if the two-cell embryo was stored at 5°C for a short period prior to culture, and the author suggested that storage of two-cell embryos at this temperature may be successful. In the present study the viability of mouse embryos after various periods at low temperature was investigated.

Methods

Two-cell mouse embryos were obtained as described by Brinster (1963). In the initial experiments, Krebs–Ringer bicarbonate (Ringer medium) was the basic medium but, in the last experiment, Dulbecco's phosphate-buffered saline was used (Dulbecco and Vogt 1954). All media contained 25 mM sodium DL-lactate, 0·25 mM sodium pyruvate, 1 mg/ml bovine serum albumin, 60 μg/ml penicillin, and 50 μg/ml streptomycin. When glycerol or dimethylsulphoxide were included, they replaced an equivalent volume of water in the medium.

For the cooling experiments, the embryos (usually 20 per treatment) were washed through two changes of Ringer medium before transfer to glass culture tubes containing 1 ml of medium. Preliminary tests had indicated that embryos did not survive for 24 hr when cooled and stored in either embryological watch glasses containing 0·5 ml of Ringer medium under 1 ml of paraffin oil or microdroplets under paraffin oil in Petri dishes. The culture tubes were surrounded by 100 ml of water and placed in a refrigerator, the rate of cooling being regulated by the initial temperature of the water jacket.

The formation of blastocysts from stored embryos following subsequent culture for 72 hr in Ringer medium (Brinster 1963) was used as a measure of viability. In most instances each experiment was repeated on four separate occasions and the results presented are the mean percentage of blastocysts cultured from the stored embryos. Prior to statistical analysis, all results were transformed to angles.

Results and Discussion

Preliminary results indicated that the inclusion of either glycerol or dimethylsulphoxide (5% v/v) in Ringer medium during storage of two-cell mouse embryos at 4–5°C for 24 hr did not improve survival irrespective of whether the embryos were transferred through stepwise increments in concentration of the compounds before cooling to 5°C. Therefore glycerol and dimethylsulphoxide were excluded from subsequent experiments examining the effects of low-temperature storage. In

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these initial experiments, shrinkage of embryos occurred on addition to and swelling on transfer from media containing glycerol or dimethylsulphoxide. The return of embryos to normal size was more rapid with dimethylsulphoxide (4–5 min) than with glycerol (1–2 hr), presumably due to the movement of dimethylsulphoxide through the cell membrane rapidly restoring osmotic equilibrium. In addition, the degree of osmotic imbalance appeared to affect survival. Whereas 22 of 40 embryos developed after exposure for 20 min to 16% dimethylsulphoxide added and removed in increments of 8%, only 3 of 40 embryos survived 20 min exposure after straight transfer to and from 16% dimethylsulphoxide.

The effects of the rate of cooling on survival at 5°C were studied by cooling embryos either at 0·1 degC/min or at 4·5 degC/min in Ringer medium (Table 1).

TABLE 1

VIABILITY OF MOUSE EMBRYOS COOLED TO 5°C AT DIFFERENT RATES AND STORED FOR PERIODS UP TO 24 HR

The response is measured as the mean percentage of two-cell embryos developing into blastocysts after storage. Values are the means of four replicates, the number of two-cell embryos cultured after cooling being given in parentheses. The mean response of uncooled controls was 51% blastocysts from 80 two-cell embryos

Rate of Cooling	Percentage of Embryos Developed after Storage at 5°C for:		
	40 min	4 hr	24 hr
4·5 degC/min	42 (59)	43 (77)	33 (75)
0·1 degC/min	47 (77)	53 (79)	20 (80)

Statistical analysis of the results showed that storage for 24 hr at 5°C depressed development during subsequent culture but that the rate of cooling did not affect this. In the next experiment, embryos were cooled at 4·5 degC/min and stored 3, 24, or 48 hr at 0, 5, or 10°C (Table 2). There was no significant effect of storage temperature but there was a linear decrease in survival with increasing time of storage. Only 7% of embryos developed into blastocysts after 48 hr compared with 54% for untreated controls.

As a medium buffered with phosphate would be more convenient for handling stored embryos than one buffered with bicarbonate, phosphate-buffered saline was compared with Ringer medium. After 24 hr at 5°C, 50% of embryos stored in the former medium developed as compared with 25% stored in the latter. In further tests, 112 of 261 embryos (43%), 46 of 158 embryos (29%), and 4 of 89 embryos (5%) developed after storage in phosphate-buffered saline for 24, 48, and 72 hr respectively. In the same experiment, 78 of 109 control embryos (72%) developed into blastocysts during culture.

As a further test of the viability of ova stored at 5°C, a total of 100 two-cell embryos from Swiss albino mice were stored for 24 hr in phosphate-buffered saline

before transfer on the first day of pseudopregnancy to the ampullae of 7 female mice (101 × C57Bl and A × C57Bl hybrids) previously mated to vasectomized C57Bl males. At 17 days, four mice were pregnant and 23 pink-eyed foetuses were obtained. This can be compared with 11 foetuses following the transfer at the same time of 16 recently collected two-cell embryos.

TABLE 2

EFFECT OF TEMPERATURE OF STORAGE ON THE VIABILITY OF TWO-CELL MOUSE EMBRYOS

Values are means of four replicates and are the percentage of two-cell embryos developed into blastocysts after cooling. The numbers of two-cell embryos cultured after cooling are given in parentheses. The mean response of uncooled controls was 54% blastocysts cultured from 72 two-cell embryos

Temperature of Storage (°C)	Percentage of Embryos Developed after Storage for:		
	3 hr	24 hr	48 hr
0	54 (71)	17 (76)	4 (72)
5	45 (78)	15 (75)	9 (78)
10	44 (77)	7 (69)	8 (66)

The results demonstrate that fertilized mouse embryos are capable of survival at 5°C for periods longer than yet reported for unfertilized mouse ova (Sherman and Lin 1959). They are not affected to any degree by temperature shock and are less susceptible to changes in the storage temperature than has been reported for other species (Chang 1947; Averill and Rowson 1959; Kardymowicz *et al.* 1963). Although survival is low compared with the best results reported for the rabbit (Hafez 1965) or the sheep (Kardymowicz, Kardymowicz, and Grochowalski 1966), this is the only study where chemically defined media, rather than serum mixtures, have been used. The use of these synthetic media is important in any critical study of environmental factors influencing survival at subnormal temperatures.

Acknowledgments

The authors are indebted to Professor C. W. Emmens for his interest and provision of facilities. This work was aided by a grant from the Australian Research Grants Committee. One of us (D.G.W.) was supported by a University of Sydney Postdoctoral Fellowship.

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