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Long-term storage of mouse embryos at —196 °C: the effect of background radiation

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SUMMARY

In order to test the feasibility of preservation of genetic stocks of mice by storage in liquid nitrogen, mouse embryos at the 8-cell stage, were frozen and stored in liquid nitrogen at −196 °C under increased radiation exposures of 1·8×, 9× and 84× background levels for periods of 6–8 months, 10–12 and 27–29 months, the 1·8× level being regarded as a control. Their survival rates to the blastocyst stage, and after transfer to recipient females, to foetal or liveborn stages were then compared with those of unfrozen or short term frozen control embryos. The freezing process *per se* caused a marked loss of viability, in comparison with the unfrozen controls, but at the 1·8× radiation level there was then no further loss in viability even at the longest storage time (27–29 months). Similarly, at the 9× radiation level there was no loss of viability during storage up to 29 months, but at the 84× level the proportions of implanted embryos and live foetuses were slightly reduced. It was not clear if this was a true effect of radiation, since it was not related to time of storage. Considering all groups, about 20–30% of the embryos originally frozen were recovered as foetuses or liveborn young. It is concluded that the preservation of genetic stocks by storage in liquid nitrogen is a feasible proposition.

1. INTRODUCTION

The preservation of mouse embryos by storage at −196 °C is a relatively new technique (Whittingham, Leibo & Mazur, 1972; Wilmut, 1972), providing a unique opportunity for the conservation of genetic material. The advantages and implications of forming embryo banks for the storage of genetic stocks have been discussed previously (Whittingham, 1974; Lyon, 1975). So far, data on survival after prolonged periods of storage are unavailable although Whittingham & Whitten (1974) recently obtained live young from embryos stored for up to 8 months at −196 °C and Leibo, Mazur & Jackowski (1974) observed no reduction in the survival *in vitro* of embryos stored for almost one year at −196 °C. Both findings suggest that mouse embryos like other tissue cells show no deterioration with time when stored at −196 °C (Meryman, 1966).

Two major factors upon which the feasibility of using storage for the preserva-

tion of genetic stocks depend are the possible damaging effects of the freezing technique *per se*, and the cumulative effect of background radiation during prolonged storage. In two of the earlier studies (Whittingham *et al.* 1972; Whittingham & Whitten, 1974) the freezing technique did not adversely affect the breeding performance of the resulting offspring, but the sample sizes were small and tests for genetic defects were not made. There are no reports that freezing itself is mutagenic (Mazur, 1975), but the cumulative effect of background radiation may possibly increase the mutation rate since no DNA repair will occur during storage at −196 °C. However, since the normal level of background radiation is only about 0·1–0·5 rad/year it has been estimated that stored embryos would have to be exposed for 50–150 years before any measurable effect could be observed, i.e. over and above the spontaneous mutation rate (Lyon, 1975). In order to assess these effects during long-term storage, we have examined the survival of 8-cell embryos exposed to levels of up to 84× background radiation during storage for up to 29 months at −196 °C.

2. METHODS

(i) *Source and collection of embryos*

In order to obtain large numbers of embryos from each female, mature 3H1 hybrid mice (F_1, C3H/HeH × 101/H) were superovulated with 10 i.u. PMSG and 10 i.u. HCG (for details see Biggers, Whitten & Whittingham, 1971). The females were mated with 3H1 males (following the injection of HCG) and $2\frac{1}{2}$ days later 8-cell embryos (F_2's) were flushed from the excised oviducts with PB1 medium (Whittingham, 1974). Eight-cell embryos were considered the most suitable stage for storage for the reasons outlined previously (Whittingham, 1975). The embryos from all females at each collection were pooled, then washed through 2 changes of PB1 medium (2 ml/wash) and 30 embryos were transferred to each plastic freezing ampoule (2 ml screw-capped ampoules, Sterilin) containing 0·1 ml PB1 medium.

(ii) *Freezing and thawing*

The procedures for freezing and thawing are fully described elsewhere (Whittingham *et al.* 1972; Whittingham, 1974). The plastic ampoules were placed in an icebath at 0 °C for 15 min before adding 0·1 ml, 3 M dimethylsulphoxide (DMSO) in PB1 medium. The final concentration of DMSO was 1·5 M. After a further 15 min the samples were transferred to the seeding bath at −6 °C. Ice formation was induced approximately 1 min later by touching the surface of each sample with sterile frozen water contained in the tip of a pasteur pipette. The caps were screwed on firmly and the ampoules attached to holders. They were transferred to the cooling bath at −6 to −7 °C (approximately 5–10 min after inducing ice formation) and cooled at rates ranging between 0·45 and 0·83 °C/min (mean 0·65 ± 0·02 °C/min) to −80 °C when they were transferred directly to liquid nitrogen for storage.

At the appropriate time intervals, samples were thawed at rates ranging between 6·0 and 11·1 °C/min (mean 8·13 ± 0·08 °C/min). At 0 °C they were placed in an ice-bath and the DMSO diluted out and the embryos recovered and washed in PB1 medium as described previously (Whittingham *et al.*, 1972).

(iii) *Radiation exposure during storage*

The frozen embryos were exposed to three different radiation dose levels (1·043, 0·112 and 0·0219 mrad/h γ-rays) by storage in liquid nitrogen refrigerators placed at different distances from a small radium source (400 μCi ^{226}Ra). The background radiation dose rate at the MRC Radiobiology Unit, Harwell, England, including cosmic rays but excluding neutrons is 0·0124 mrad/h and therefore the radiation dose levels in the refrigerators corresponded approximately to 84×, 9× and 1·8× the background dose level. The 1·8× background dose level was regarded as a control, since such a level is within the range of background doses found at different sites.

The frozen samples resulting from each embryo collection were randomly distributed among the three refrigerators, care was taken to label the ampoules and record (in duplicate) the refrigerator, can and cane positions. The liquid nitrogen was kept at a constant level and the refrigerators partially rotated weekly so that the ampoules were uniformly exposed to the radium source. The refrigerators were connected to an alarm system which would indicate if any undue loss of liquid nitrogen occurred. Plastic ampoules were chosen for storage to obviate the likelihood of slight variation in radiation dose within similar glass ampoules. Embryos were thawed and recovered after exposure to the different levels of radiation for 6–8, 10–12 and 27–29 months. At the highest radiation level, this was equivalent in radiation dose to 42–56, 70–84 and 189–203 years respectively.

(iv) *Embryo recovery and culture*

Depending upon the number of pseudopregnant recipients available at one time, an appropriate number of ampoules from each radiation level were thawed the day before transfers were to take place. After thawing, the embryos from each ampoule were washed free of DMSO and their numbers and morphological appearances were recorded, the morphological categories scored being normal, damaged with 1 or more lysed blastomeres, swollen, or totally degenerated. Loss of or damage to the zona pellucida was noted too. Afterwards, they were placed in drops of a mouse embryo culture medium (No. 16, Whittingham, 1971) contained in a plastic petri dish with a paraffin oil overlay, and were cultured for 20–24 h (for details see Biggers *et al.*, 1971). The number of blastocysts and morulae that were normal in appearance was then again recorded immediately before transfer to a recipient female.

(v) *Embryo transfer*

Mature 3H1 females were naturally mated with sterile males, (carrying the translocation T145H) and 4–6 embryos were transferred to each uterine horn on the third day of pseudopregnancy (day 1 is the day on which the vaginal plug is found). Each female received embryos from 1 radiation dose level, and for each dose level the females were randomly divided into two groups. One group were killed and examined on day 14 or 15 of gestation and the number of normal live embryos and resorbing implantation sites (moles) recorded. The other group were allowed to litter and the resulting progeny screened for any abnormalities.

(vi) *Statistical analysis*

The data on survival after freezing and following transfer was compared by χ^2 analysis both for individual comparisons and to test for interaction between radiation level and storage time. This type of analysis was considered most suited to the data which were accumulated, for practical reasons, over a considerable period in time.

3. RESULTS

Over 90% of the superovulated females mated successfully and an average of approximately 25, 8-cell embryos per mated female were recovered (Lyon, 1975). During the course of the experiment more than 7000, 8-cell embryos were collected; the majority were frozen and distributed randomly among the three radiation dose levels. The remainder served as controls; one group was frozen and stored at −196 °C for 24 h before thawing, culture and subsequent transfer, the other group was not frozen but only cultured before transfer.

Table 1. *A comparison of the development* in vitro *of unfrozen 8-cell mouse embryos with frozen-thawed 8-cell embryos previously stored at −196 °C for 24 h*

Pre-treatment	No. of samples thawed	No. of embryos recovered/total no. frozen (%)	No. of embryos normal at recovery (%)	No. of morulae and early blastocysts after 20–24 hr culture (%)
None	—	—	240	230/240 (95·8)
Frozen–thawed	12	327/360 (90·8)	187 (57·2)	178 (54·4)

(i) *Recovery and development* in vitro

Table 1 compares the development of freshly collected control 8-cell embryos to the morulae and early blastocyst stage with the development of embryos previously stored at −196 °C for 24 h before thawing and culture. Similar proportions of fresh and frozen–thawed embryos, normal at recovery, developed

to morulae and early blastocysts (96% versus 178/187 or 95%). The overall survival rate of the frozen embryos on thawing (54%) was lower than previously reported; this was due to storage in plastic instead of glass ampoules (see Discussion).

Table 2 summarizes the recovery and development of frozen–thawed 8-cell mouse embryos exposed to the three different radiation dose levels for varying periods of time. A total of 101 samples were thawed (3030 embryos), 2739 (90·4%) were recovered, 1324 (48·3%) of these appeared morphologically normal at recovery, 852 (31·1%) were damaged, i.e. with one or more blastomeres lysed, 222 (8·1%) were swollen and 341 (12·4%) were degenerated. Less than 3% (78) had lost their zona pellucida and the majority of these (71) were in the damaged category. The only group which differed significantly from the rest in the proportions of normal and degenerated embryos on recovery was the 84× background level of radiation after 6–8 months storage. Since no other comparison (χ^2) was found to be significantly different and also there was no evidence of an interaction between radiation level and storage, the lower responses observed at the highest radiation level after 6–8 months storage are probably due to variables other than radiation and/or storage time.

In Table 2, the number of 8-cell embryos reaching the morula and blastocyst stage without any observable cell damage after 20–24 h in culture is given for the various treatments. In some groups the slightly higher numbers of embryos reaching the morula and blastocyst stage compared to the numbers scored as morphologically normal on recovery is due to the subsequent recovery of the embryos originally classified as having swollen or distended blastomeres. Intracellular damage may have impaired the normal rapid removal of DMSO during the dilution and washing procedure following thawing. Overall, the numbers developing to morulae and early blastocysts were similar to the numbers scored as morphologically normal on recovery in all treatments. There were no significant differences between storage time and radiation dose level in the proportions of embryos recovered, normal embryos at recovery and embryos developing to the morula and early blastocyst stage.

(ii) *Development* in vivo *after transfer*

The development of control embryos (fresh embryos and embryos previously stored at −196 °C for 24 h) following transfer is summarized in Table 3. A total of 314 morulae and early blastocysts were transferred to 29 pseudopregnant recipients (mean 10·8 embryos per female) which all became pregnant. Seventeen females were examined on day 14 or 15 of gestation and a total of 167 implantation sites (88·4%) and 120 normal live foetuses (63·5%) were found. The remaining 12 females were allowed to litter and produced a total of 70 young (56·0%, 32♀♀ and 38♂♂). No significant differences were found between the survival of unfrozen and frozen–thawed control embryos following transfer either to day 14 or 15 or to birth. The development of frozen–thawed embryos exposed to three different radiation dose levels during storage is summarized in Table 4. A total of

Table 2. *Recovery and development* in vitro *of frozen-thawed 8-cell mouse embryos following exposure to three different levels of background radiation for varying periods of time during storage*

Approx. level of background radiation	Length of exposure (months)	No. of samples thawed	Mean % embryos recovered/sample (± S.E.)	Mean % embryos normal at recovery/sample (± S.E.)	Mean % morulae and early blastocysts/sample after 20–24 h culture (± S.E.)
1·8 ×	6–8	9*	89·63 ± 2·86	47·51 ± 4·46	57·69 ± 2·67
	10–12	10	91·33 ± 2·54	51·61 ± 4·07	53·92 ± 4·72
	27–29	12	89·16 ± 2·91	47·32 ± 3·39	49·82 ± 3·49
9 ×	6–8	9	88·51 ± 3·93	54·15 ± 5·15	55·99 ± 3·57
	10–12	10	91·33 ± 2·12	50·38 ± 3·24	52·97 ± 2·74
	27–29	13	91·03 ± 1·62	45·97 ± 4·44	53·18 ± 3·45
84 ×	6–8	9	89·63 ± 4·02	39·55 ± 5·98	48·28 ± 4·94
	10–12	15	91·11 ± 3·20	47·33 ± 3·46	47·90 ± 4·14
	27–29	14	90·95 ± 2·02	47·89 ± 3·15	55·14 ± 2·75

* Thirty embryos/sample.

Table 3. *Post-implantation development of unfrozen and frozen-thawed 8-cell mouse embryos cultured for 20–24 h before transfer to the uterine horns of day 3 pseudopregnant recipients*

Pre-treatment	Total no. of embryos transferred (no. recipients)	No. of embryos transferred to pregnant recipients (no. of pregnant ♀♀)	Examination at day 14 or 15 of gestation: No. of implantation sites (%)	Examination at day 14 or 15 of gestation: No. live foetuses (%)	No. of live young at birth (%)	Live foetuses and liveborn (%)
None	195 (17)	121 (10)	111 (92)	81 (67)	—	64
		74 (7)	—	—	44 (59)	
Stored at −196 °C for 24 h	119 (12)	68 (7)	56 (82)	39 (57)	—	55
		51 (5)	—	—	26 (51)	

Table 4. *Post-implantation development of frozen-thawed 8-cell mouse embryos, exposed to three different levels of background radiation during storage and cultured for 20–24 h before transfer to the uterine horns of day 3 pseudopregnant recipients*

Approx. level of background radiation	Length of exposure (months)	Total no. of embryos transferred (no. of recipients)	No. of embryos transferred to pregnant recipients (no. of pregnant ♀♀)	Examination at day 14 or 15 of gestation		No. of live young at birth (%)	Live foetuses and liveborn (%)
				No. of implantation sites (%)	No. of live foetuses (%)		
1·8 ×	6–8	132 (11)	76 (6)	72 (95)	45 (59)	—	47
			56 (5)	—	—	17 (30)	
	10–12	124 (10)	60 (5)	60 (100)	40 (67)	—	65
			64 (5)	—	—	40 (63)	
	27–29	164 (16)	67 (6)	55 (82)	29 (43)	—	45
			76 (8)	—	—	35 (46)	
9 ×	6–8	142 (12)	83 (7)	65 (78)	40 (48)	—	47
			59 (5)	—	—	27 (46)	
	10–12	120 (10)	59 (5)	53 (90)	42 (71)	—	67
			61 (5)	—	—	38 (62)	
	27–29	189 (16)	95 (8)	80 (84)	47 (49)	—	52
			94 (8)	—	—	51 (54)	
84 ×	6–8	102 (10)	49 (5)	30 (61)	18 (37)	—	42
			53 (5)	—		25 (47)	
	10–12	170 (14)	85 (7)	71 (84)	38 (45)	—	49
			49 (4)	—	—	28 (57)	
	27–29	221 (18)	90 (7)	64 (71)	34 (38)	—	45
			106 (9)	—	—	55 (52)	

1364 embryos were transferred to 117 pseudopregnant recipients (mean 11·7 embryos per female), one female died postoperatively and 110 (94·8 %) subsequently became pregnant. Fifty-six females were examined on day 14 or 15 of gestation and a total of 550 implantation sites (82·8 %) and 333 normal live foetuses (50·2 %) were found. The remaining 54 females were allowed to litter and produced a total of 316 young (51·1 %, 181 ♀♀ and 135 ♂♂).

The interaction between radiation dose and storage time was tested separately by χ^2 analysis for the proportion of implants, foetuses and live young, but no evidence for interaction of any of these variables was found. Although the proportions of implants and live foetuses show a significant linear decrease from the lowest to highest radiation dose levels at 6–8 ,and 10–12 months (for combined storage times, $P < 0{\cdot}001$ for both implants and live foetuses), there was no significant decrease after 27–29 months storage. The lowest implantation rate (61 %) was at 84 × background after 6–8 months storage differing significantly from all other treatments ($P < 0{\cdot}01$). In addition, the overall proportions of foetuses and live-born were lower at all radiation levels examined after 6–8 months and 27–29 months than after 10–12 months storage. The reasons for the lower responses are not obvious, because of the extended period over which the experiments were conducted but possibly they may be caused by variations in culture conditions, age of recipients, transfer technique or time of year when embryos were transferred.

The ratio of live foetuses to implantation sites on day 14 or 15 of gestation was similar with the exception of the 84 × background radiation dose level at 6–8 months ($P < 0{\cdot}01$) and the unfrozen controls ($P < 0{\cdot}01$). Therefore, it was concluded that early post implantation death of embryos was not affected by the level of radiation and/or storage time.

4. DISCUSSION

The present results show that there is no apparent decrease in viability of mouse embryos stored at − 196 °C for up to 2¼ years when exposed to basal levels of background radiation. Previously, no decrease in viability of mouse embryos was found after storage for up to 1 year (Whittingham & Whitten, 1974; Leibo *et al.* 1974). These results are in agreement with the preservation of other tissue cells at − 196 °C where no deterioration in viability has been observed after prolonged periods of storage (Meryman, 1966).

The overall survival rate on thawing (the total numbers of embryos appearing morphologically normal at recovery was approx. 50 %) was much lower than previously reported although the rates of cooling and thawing were within the ranges for optimal survival (Whittingham *et al.*, 1972). The lower response was apparently due to storage of embryos in plastic ampoules, since a direct comparison of the survival of 8-cell embryos stored in plastic and glass ampoules showed that 59 % (129/219) and 74 % (156/211), respectively, were normal at recovery and 56 % (123/219) and 72 % (152/211), respectively, developed to blastocysts after 48 h *in vitro* (Whittingham, 1976). Variations in the transmission

of heat through the plastic may have significantly altered the rates of cooling and thawing, producing sub-optimal rates in the immediate vicinity of the embryos. However, such variations were not detectable by the thermocouple temperature recorder. The plastic ampoules were chosen in order to give a greater uniformity of radiation dose than afforded by similar glass ampoules.

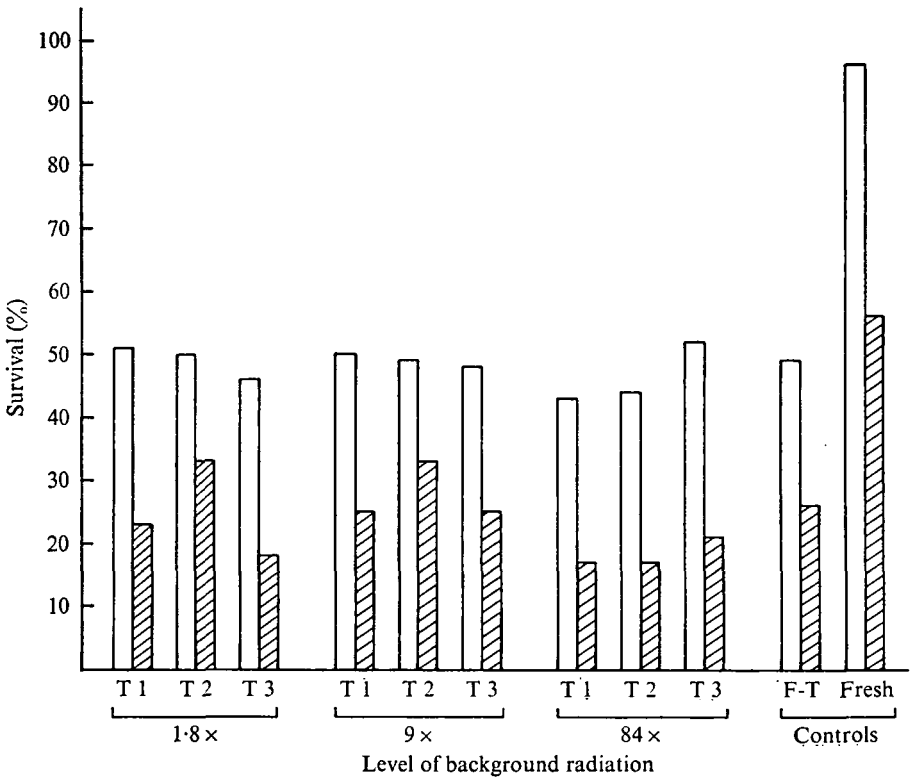

Fig. 1. Survival of 8-cell mouse embryos to morulae and early blastocysts (□) and foetuses and liveborn (▨) expressed as a percentage of the number of embryos originally frozen. T 1, T 2 and T 3 represent 6–8, 10–12 and 27–29 month exposures to the three radiation dose levels. F/T Frozen–thawed control embryos stored at −196 °C for 24 h. Fresh, Unfrozen control embryos.

When the background level of radiation was increased ninefold (equivalent to a radiation dose of 0·90 rad/year) no adverse affect was found on embryo viability during storage for up to 2¼ years. However, the exposure of embryos to 84× the background level of radiation, while not appreciably affecting survival immediately upon thawing, significantly reduced the overall implantation rate, and numbers of live foetuses on examination at day 14 or 15 of gestation, at 6–8 and 10–12 months but not at 27–29 months. Nevertheless the implantation rate was lower at 27–29 months.

The proportion of live foetuses to implantation sites was similar to all other treatments and the controls, indicating that loss of embryos at the highest

radiation dose occurred prior to implantation. The additive effects of slightly lower numbers of normal embryos after thawing and the reduced numbers implanting accounts for this loss. Assuming that the maximum estimated radiation dose for embryos exposed to 9× the background level of radiation for 27–29 months is 2·4 rad and the maximum estimated dose at 84× background level after 6–8 months storage is 5·8 rad, then the radiation dose at which early embryonic death becomes apparent occurs somewhere between 2·4 and 5·8 rad.

Whether this is a real effect is not clear, since there is no significant increase in lethality with the increase in exposure time at the highest radiation level and no interaction was found with radiation level and time of storage. Also, the LD_{50} for acutely X-irradiated 8-cell embryos *in vitro* (unfrozen) is several orders of magnitude higher (388–473 rad – Goldstein, Spindle & Pedersen, 1975; Dufrain, 1975). Furthermore there is no indication of an increase in malformation in the foetuses and live-born obtained from embryos stored at the highest radiation level.

To evaluate the efficiency of the storage technique, the viability of stored embryos in each treatment is summarized in Fig. 1. The numbers of morulae and early blastocysts after culture and the numbers of foetuses and liveborn after transfer are expressed as percentages of the total numbers of embryos originally frozen in each group. Apart from the major differences and similarities between treatments (discussed above), there are two major sources of embryonic loss common to all treatments. Firstly, in all the frozen groups, including the 24 h group, between 48 and 57 % of the embryos fail to reach the morula and blastocyst stage, whereas the unfrozen controls showed very little drop. This loss is thus inherent in the freezing technique itself. Secondly, a further 16–31 % fail to develop into foetuses and liveborn and this loss occurs mainly in the early post implantation period (see Table 4). While the reduction of the early post implantation losses may be impracticable (see unfrozen controls, Table 3 and Figure 1), improvements in survival after freezing by the use of glass ampoules could possibly increase survival of frozen-thawed embryos at term by approximately 20 % (overall survival 45–55 %).

In conclusion, embryo storage is a feasible proposition. No evidence for reduced viability after periods of storage for over 2 years was found. The survival rates to term are high enough to enable breeding stocks to be re-established from frozen embryos. The hazard of background radiation during storage appears negligible although the effect observed at the highest radiation dose still needs further examination during longer periods of storage. The genetic risk encountered by radiation during storage in glass ampoules with DMSO as the cryoprotective agent would be even further reduced since glass, DMSO and low temperature itself afford some radiation protection (Ashwood-Smith, 1967; Nias & Ebert, 1969). In the light of these findings, it is hoped that embryo banking will now become a practical routine procedure.

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