Further studies on the effect of radiation during the storage of frozen 8-cell mouse embryos at -196°C

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Summary. Frozen 8-cell mouse embryos were treated with radiation doses of 0, 10, 50, 100 or 200 cGy γ -rays at a dose rate of ≈ 5 cGy/day. After thawing the embryos were scored for normal morphological appearance and for development to morulae and blastocysts after 24 h in culture. Embryos from each treatment were then separately transferred to the uteri of pseudopregnant foster mothers which were killed at Day 14 of pregnancy. There was no effect of radiation on morphological appearance, development to morulae and blastocysts, implantation rate, or on the ratio of live fetuses to the number of transferred embryos.

As there appeared to be no detrimental effect of up to 200 cGy on frozen 8-cell mouse embryos and, as this is the equivalent of ≈ 2000 years of background radiation, it is concluded that normal levels of background radiation would not be a hazard to the long-term storage of mammalian embryos.

Introduction

In previous experiments designed to examine the cumulative effects of background radiation during the storage of mouse embryos at -196°C (Whittingham, Lyon & Glenister, 1977; Lyon, Whittingham & Glenister, 1977) there appeared to be a decrease in viability, as measured by the ability of the embryos to develop to the blastocyst stage *in vitro*, after storage for about 5 years at the higher radiation doses (Lyon, Glenister & Whittingham, 1981). This was a surprising finding since even the highest radiation dose was relatively small (reported as ~ 64 cGy) and well below the dose of X-irradiation that prevented 50% of 8-cell mouse embryos developing to the blastocyst stage when administered at normal temperatures (388–473 R: Goldstein, Spindle & Pedersen, 1975; Dufrain, 1976). It became even more surprising when it was realized that, owing to circumstances beyond our control, the actual radiation doses received were lower than reported in our paper. In addition, low temperature and cryoprotective agents such as dimethylsulphoxide (Me_2SO) and glycerol are known to afford some protection against radiation damage (Ashwood-Smith, 1967; Nias & Ebert, 1969). Damage induced in somatic CHO cells X-irradiated at -196°C in the presence of 10% Me_2SO was reduced by a factor of 3.7 when compared with similar cells irradiated at $+22^{\circ}\text{C}$ (Ashwood-Smith & Grant, 1977). Indirect evidence has shown that preimplantation mouse embryos are capable of repairing DNA damage induced by ultraviolet light but the amount of damage that can be repaired is unknown (Pedersen & Brandiff, 1981). It is possible that the activity of repair enzymes is reduced by freezing and thawing and, irrespective of the radio-protection afforded by low temperature and cryoprotectants, there is insufficient enzyme activity to repair effectively the radiation damage accumulated during storage.

This apparent effect on the ability to develop to the blastocyst stage *in vitro* was the only effect of radiation detected in the previous studies. Other criteria examined were: post-implantation development to fetuses and liveborn, breeding tests on the liveborn young to assess their fertility and to look for mutations amongst their offspring, chromosome preparations from the testes of the male liveborn and dissection of female liveborn in mid-pregnancy to detect dominant lethal mutations (Lyon *et al.*, 1981).

The major uncertainty arising from the earlier experiments is whether the decrease in viability observed at the higher radiation doses was due to radiation *per se* or some other uncontrolled factor. In an attempt to solve this problem, a further short-term experiment was carried out to examine the survival of 8-cell mouse embryos after exposure at -196°C to various doses of γ -rays administered at a dose rate of ~ 5 cGy/day.

Materials and Methods

Embryo collection

Embryos were obtained from 6–8-week-old 3H1 (F_1 , C3H/HeH $\times$ 101/H) hybrid mice which were induced to superovulate with gonadotrophins (for details see Biggers, Whitten & Whittingham, 1971). After the injection of hCG, the females were mated with 3H1 males and 8-cell embryos were flushed from the excised oviducts 3 days later with a Hepes-buffered embryo culture medium (M2: Quinn, Barros & Whittingham, 1982). At each collection the embryos from all females were pooled and washed through several changes of Medium M2 (2 ml/wash). Finally, 30 embryos were transferred to each glass ampoule (2 ml capacity; Jencons, Leighton Buzzard LU7 8UA, U.K.) containing 0.15 ml Medium M2 and the ampoules were then placed in an ice-bath at 0°C .

Freezing and thawing

The embryos were frozen and thawed by the 'rapid' method previously described (Whittingham, Wood, Farrant, Lee & Halsey, 1979). After holding the embryos for 15 min at 0°C , 0.15 ml 3 M-dimethylsulphoxide was added to achieve a final concentration for cryoprotection of 1.5 M-Me₂SO. The ampoules were sealed and 15 min later transferred to a 'seeding' bath at -9°C . After 2 min in the seeding bath, ice formation was induced in the samples by sharply tapping the ampoules with metal forceps. The samples were then transferred to a cooling bath at -6°C and they were cooled at rates ranging between 0.3 and 0.6°C per min. On reaching -35°C the ampoules were plunged directly into liquid nitrogen for storage. All samples were thawed rapidly ($\sim 500^{\circ}\text{C}/\text{min}$) by agitating the ampoules in a water bath at 40°C . As soon as all the ice had disappeared the ampoules were placed on the bench at room temperature for 5 min before adding 1 ml Medium M2 to each ampoule. The embryos were recovered, washed through several changes of Medium M2 and placed in drops of embryo culture medium (No. 16: Whittingham, 1971) overlaid with paraffin oil. They were cultured for 20–24 h at 37°C in an atmosphere of 5% CO_2 in air.

Radiation exposure during storage

During storage in liquid nitrogen the embryos were irradiated from a gamma source (^{60}Co) at a dose rate of 5.14 cGy/day. The ampoules from each embryo collection were randomly allocated to one of 5 different total doses of γ -radiation (0, 10, 50, 100 and 200 cGy γ -rays). After receiving the appropriate dose of radiation, the ampoules were removed from the radiation field and stored at normal levels of background radiation until thawed. The total length of storage at -196°C varied between 5 and 8 months.

Embryo recovery and culture

Thawing took place on 3 occasions over a period of 14 days. The ampoules were coded according to the dose of radiation, to avoid any possible bias in scoring embryos after recovery, culture and transfer. After thawing, the morphological appearance of the embryos was recorded, i.e. the number normal, damaged (1 or more blastomeres lysed), swollen or totally degenerated. After 20–24 h in culture the number of embryos reaching the morula and blastocyst stages was recorded. At this time the embryos from ampoules with the same code were pooled together in Medium M2 and held at room temperature in preparation for transfer.

Embryo transfer

Mature 3H1 females were naturally mated with sterile males (carrying the translocation T145H) and between 4 and 8 embryos were transferred to each uterine horn on the afternoon of the 3rd day of pseudopregnancy (Day 1 is the day on which the vaginal plug is found). Each female received embryos from only one dose level of radiation. All females were killed on Day 14 of gestation and the numbers of normal live fetuses, dead fetuses and resorbing implantation sites (moles) were recorded.

Statistical analysis

After decoding, the data on embryonic survival after freezing and thawing, culture and transfer were compared by χ^2 analysis to test for homogeneity within and between each radiation dose and each thawing time.

Results

Over a period of 3 months 1200 8-cell embryos from 10 separate collections were frozen and stored at -196°C in glass ampoules each containing 30 embryos. Within 3–4 months of the completion of γ -irradiation the samples were thawed on 3 separate occasions over a 2-week period. Table 1 summarizes the recovery and development *in vitro* of frozen-thawed 8-cell embryos after exposures to the different doses of γ -irradiation during storage. Of the 1200 embryos (40 samples) thawed, 1110 embryos (93%) were recovered and 723 (60%) appeared morphologically normal when placed in culture after removal of the cryoprotective agent. A total of 794 (66%) had reached the morula or early blastocyst stage 24 h later. The slight increase in the number of embryos reaching the morula and blastocyst stages was due to the inclusion of embryos which originally appeared swollen after recovery and embryos which had only lost 1 or 2 blastomeres during freezing and thawing. There

Table 1. Survival and development *in vitro* of frozen-thawed 8-cell mouse embryos after exposure to various doses of γ -irradiation during storage at -196°C

Radiation dose (cGy)	No. of ampoules* thawed	No. of embryos recovered (%)	No. of embryos normal at recovery (%)	No. of morulae and blastocysts after 24 h in culture (%)
0	8	225 (93.8)	135 (56.3)	160 (66.7)
10	8	223 (92.9)	142 (59.2)	151 (62.9)
50	8	222 (92.5)	147 (61.3)	157 (65.4)
100	8	223 (92.9)	155 (64.6)	172 (71.7)
200	8	217 (90.4)	144 (60.2)	154 (64.2)

* Each ampoule contained 30 8-cell embryos.

All percentages calculated from total no. of embryos originally frozen.

Table 2. Post-implantation development of frozen-thawed 8-cell mouse embryos, exposed to different doses of γ -irradiation during storage, and cultured for 20–24 h before transfer to Day-3 pseudopregnant recipients

Radiation dose (cGy)	No. of embryos transferred to pregnant recipients (no. of pregnant ♀♀)	Examination on Day 14 of gestation		
		No. of implantations (%)†	No. of live fetuses (%)†	No. of live fetuses (%) no. of implantations
0	148 (12)	130 (87.8)	81 (54.7)	62.3
10	151 (12)	119 (78.8)	84 (55.6)	70.6
50	160 (13)	147 (91.9)	98 (61.3)	66.7
100	154 (13)*	135 (87.7)	82 (53.2)	60.7
200	147 (12)	140 (95.2)	80 (54.4)	57.1

* In addition one recipient, which received 14 embryos, failed to become pregnant.

† Percentages calculated from no. of embryos transferred.

was no evidence to suggest that survival or subsequent development to the morula and blastocyst stages was affected by the amount of radiation given during storage. In addition, survival and development *in vitro* were not related to the separate freezing and thawing times.

The post-implantation development of embryos from the various treatments after transfer to pseudopregnant recipients is summarized in Table 2. As many embryos as possible were transferred, but the numbers were limited by the availability of recipients. Also, in the 50 cGy group, 3 embryos, initially classified as slightly abnormal, cavitated during the holding time on the bench and were deemed fit for transfer.

A total of 774 embryos was transferred to 63 pseudopregnant recipients; 62 (98%) females containing a total of 673 (89%) implantations were examined on Day 14 of gestation and 425 (56%) normal live fetuses were dissected from the implantation sites. Again, the dose of radiation did not affect the proportion of transferred embryos developing into normal live fetuses at 14 days gestation. Post-implantation development was also unrelated to the time of thawing. When the proportion of live fetuses to the total number of implantation sites was compared, the number of embryos lost during early post-implantation appeared to increase with increasing radiation dose, i.e. from 29 to 43% between 10 and 200 cGy. However, since the loss in the control unirradiated group was at an intermediate level (38%) it is doubtful whether this decrease in viability was due to the effects of radiation. Statistical analysis showed that the 5 observations were satisfactorily homogeneous ($\chi^2_4 = 6.16$; $P = 0.19$).

In Text-fig. 1, a summary is given of the proportion of embryos originally frozen surviving to the morula and blastocyst stages *in vitro*, implanting, and developing to live fetuses at Day 14 of

Text-fig. 1. Survival of 8-cell mouse embryos after exposure to various doses of γ radiation during storage at -196°C and expressed as a percentage of the number of embryos originally frozen and thawed at each dose (240 embryos per dose).

gestation after transfer. About 35% of the total number of embryos frozen in all treatments developed into normal live fetuses and there was no significant difference in proportions surviving at each radiation dose.

Discussion

The irradiation of 8-cell mouse embryos during storage at -196°C with doses of up to 200 cGy had no adverse effect on the subsequent survival of the thawed embryos. The reason for the decrease in survival of embryos stored for about 5 years at -196°C and receiving 4.5 and 42 cGy of γ -irradiation is puzzling (Lyon *et al.*, 1981): it may have been due to uncontrolled factors resulting from the long time course of the experiment or to the decrease in the activity of DNA repair enzymes during prolonged storage. Since all biological activity in cells ceases below -130°C it seems unlikely that embryos would deteriorate during prolonged storage at -196°C . In the previous study (Lyon *et al.*, 1981), it is possible that a larger proportion of embryos was frozen at a stage of the cell cycle more susceptible to radiation damage. Dufrain & Casarett (1975) have shown an increased sensitivity to radiation between the second and third cleavage division and a similar sensitivity may occur between the third and fourth cleavages. However, the low doses of radiation that were used in the long-term studies and the increased radioprotection afforded to cells during low-temperature storage (Ashwood-Smith & Grant, 1977) would make it extremely difficult to measure any minor changes in radiosensitivity occurring between cleavage divisions in embryos stored at -196°C .

In the present study, the rapid freezing and thawing method was used (Whittingham *et al.*, 1979) and the embryos were stored in sealed glass ampoules instead of screw-cap plastic ampoules. It is possible that the increased amount of water remaining in the embryos after rapid freezing may have decreased the sensitivity of embryos to radiation, although for seeds stored at temperatures above 0°C susceptibility to radiation damage is greater in the hydrated form than when they are dehydrated (Roberts, 1972). Glass will provide a small amount of protection against radiation but, more significantly, the survival of embryos after freezing and thawing in glass ampoules is much higher than in plastic ampoules, as shown previously (Whittingham, 1976). This probably accounts for the overall increase in survival rates to the morula and blastocyst stages *in vitro* and the number of implantations and live fetuses after transfer (Text-fig. 1) when compared with the long-term study (Whittingham *et al.*, 1977; Lyon *et al.*, 1981).

Assuming that the background level of radiation at Harwell is ≈ 0.1 cGy/year, embryos irradiated at the highest dose (200 cGy) received the equivalent of 2000 years of background radiation. Since no evidence of radiation damage was detected at this dose in the present study and it seems unlikely that the decreased viability observed at low radiation doses in the earlier long-term study was due to radiation, it is concluded that normal background radiation would not be detrimental to the long-term storage of mammalian embryos.

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