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Survival of Electrical Activity of Deep Frozen Fetal Mouse Hearts After Microwave Thawing

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Long-term preservation of organs is likely to be achieved only in the frozen state. Certain multicellular structures have already been successfully preserved after freezing. Thus, chick embryo heart anlage survived freezing for short periods in liquid nitrogen (6) using ethylene glycol (EG) as a cryoprotective agent. Whittingham *et al.* (18) froze 2–8 cell whole mouse embryos to −196° and −269° C at slow cooling rates (0.3° to 20° C/min) and then thawed them slowly at rates of 4° to 25° C/min, with subsequent development of 50–70% of the embryos into blastocyst on culture. When these were placed in pseudo pregnant mothers, 65% became implanted as pregnancies, and of these over 40% became fetuses or went to term. Dimethylsulfoxide (DMSO), at 1 M concentration, was about twice as effective as an equal concentration of glycerol. There was an optimum cooling rate and a probable need for slow as well as controlled thawing rates.

Supercooled adult hearts have resumed beating (1, 10, 15, 16) but prior to the work of Offerijns and Krijen in 1972 (12) and Rapatz in 1970 (14), attempts to freeze adult mammalian hearts had met with little success. A detailed review of the subject has been given by Luyet (11). Rapatz (14) obtained partial resumption of activity in all parts of adult frog hearts after freezing to below −55° C, using ethylene glycol as a cryoprotective agent. Offerijns and Krijen (12) added DMSO to perfusates of isolated adult rat hearts. With supercooling to temperatures not below −18° C, all hearts survived in 2.1 M DMSO. With freezing to −30° C, young rat hearts (10–16 days old) also recovered, but older hearts did not.

This paper reports our experience with freezing of the fully differentiated fetal heart of the mouse, which can be reimplanted in the ear of an adult syngeneic mouse and studied electrically over a long period of time. Microwave energy (at 2450 MHz) was used as one method of thawing, a method which provides uniform heating of the medium in which hearts were frozen, and at rates that can be controlled. This thawing technique may well be essential for larger organs, as it may be the only way to achieve uniform heating.

MATERIALS AND METHODS

Heart transplant. Hearts were removed from Balb-c fetuses obtained at 17–19 days gestation. The hearts at this time measure approximately 1 mm in diameter and beat rhythmically. Following thawing, the embryonic hearts were implanted directly into the ear of adult syngeneic mice anesthetized by nembutal. The anterior aspect of the ear was injected subcutane-

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ously with 0.1 ml of saline. The injection causes the two layers of skin to separate and thus form a pocket for the fetal heart. A small incision was made to introduce the fetal heart into the subcutaneous space. Fetal hearts implanted in this manner are nourished by the surrounding tissue fluid until an abundant capillary supply is formed a few days after grafting (3, 5, 8, 9). Dimethylsulfoxide was not removed before implantation in the ear as it was felt that it would not harm the tissue in diluted concentration.

Freezing. The fetal hearts were placed in one of four prechilled solutions: solution A: Eagle's minimal essential medium (MEM) containing Hepes buffer, 10% (v/v) fetal calf serum, and 10% (v/v) DMSO; solution B: McCoy's 5a medium containing Hepes buffer, 10% (v/v) fetal calf serum, and 10% (v/v) DMSO; solution C: Cross solution (4), containing 10% (v/v) DMSO (but no fetal calf serum or other proteins); and solution D: the same as solution A without DMSO. The final composition of solutions A, B, or C with respect to DMSO was obtained by slowly adding DMSO, after the hearts had been placed in the DMSO-deficient solutions. Dimethylsulfoxide was added over a 20 min period to give the final concentrations if 10% (v/v). Five milliliter glass vials of the various solutions, each containing one heart, were then immediately placed in a prechilled freezing unit (Linde BF-4-1). The freezing rate was maintained between 0.5° and 0.7° C/min, by the regulated thermocouple control system, down to −100° C. The samples were then placed in liquid nitrogen vapor and cooled at 5°–10° C/min down to −196° C. They were stored for 72–216 hr at −196° C before being rewarmed.

Thawing process. Frozen hearts were rewarmed either by placing the 5 ml vials in a 25° C water bath, thereby raising the temperature at a rate of ∽150° C/min, or by a microwave system, similar to that recently reported for thawing tissue culture cells at different rates (17). In the microwave system, frozen samples were rotated on a Teflon arm attached to an oscillating shaft in the resonant cavity, movement in each plane being powered by motors external to the cavity. Power was fed to the cavity by means of a stripline radiator from a 2450 MHz magnetron, the power output of which could be varied from 0.4 to 2.0 kW. The cubic cavity was designed to resonate in a large number of modes (7). Four samples were thawed at a time. Additional stationary water samples were included in the cavity to protect the magnetron. Although a high percentage of the energy was still reflected back to the magnetron, the technique provides a useful method for uniform heating of samples of different shapes and size. In these experiments, mean heating rates of 200° C/min were used to thaw the hearts from −196° to 10° ± 10° C.

Assay of graft function. The graft could not be assessed as a pump; however, electrical property was evaluated by electrocardiography, and contractility could be seen directly through the skin of the external ear with a magnifying glass. The leads of the ECG machine were attached to the anesthetized test animal by means of small clips. A small clip was also attached to the mouse ear containing the fetal heart and connected proximally to the V-lead or search electrode of the machine. As seen in Figure 1, the electrical activity of both the adult heart (b) and that of the fetal heart (a) can be recorded simultaneously as two distinct sets of rhythmic electrical activities. For a detailed description of the technique, see Jirsch, *et al.* (8).

RESULTS

As controls, Balb-C mice received direct syngeneic ear transplants of unfrozen fetal

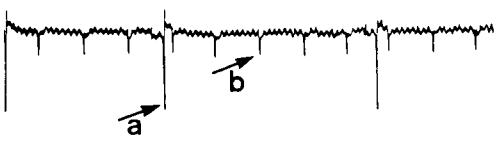

FIG. 1. A typical ECG showing normal mouse complexes (arrow b) at approximately 250 beats/min with slower superimposed fetal heart rhythm (arrow a) at one-fifth the adult rate.

hearts: 95% of these control transplants had electrical activity by the 5th day and continued to function for periods in excess of 90 days. In previous experiments (8), electrical activity of similar transplants had existed for periods in excess of 1 year.

The recovery of electrical activity for the frozen-thawed hearts is shown in Table 1. Such activity was monitored at intervals over the period of 6 to 30 days after implantation. As in the control groups, those hearts which had survived the initial period after grafting continued to beat indefinitely, i.e., up to 100 days at the time of writing. There were no differences in electrical activity between control implants and those which had survived freeze-thawing, when analyzed for the nature of the electrical complexes or their rate.

The results show that solutions A and B, Eagle's MEM or McCoy's 5a, both also containing 10% fetal calf serum, 10% DMSO, and Hepes buffer, permit significant survival, in contrast to solution C (Cross solution with 10% DMSO but not containing Hepes buffer or fetal calf serum) and solution D (Eagle's MEM, with fetal calf serum and Hepes buffer but not containing DMSO). Thus, if the results for solution A and B are pooled, the overall survival between 6–15 days is 37 of 55, or 67%, and at more than 30 days is 32 of 54, or 59%. No survival was obtained with solution C or D.

From these results, we conclude (a) that both DMSO and fetal calf serum may be important components for survival of fetal mouse hearts exposed to freeze-thaw injury; (b) that microwave thawing is as effective as thawing in a water bath and encourages the hope that it may be effective with larger tissues where water bath thawing is ineffective; and (c) that this model can be used, in future work, to assess different cryoprotective agents and freeze-thawing techniques on survival of a multicellular organ which is at the upper limit of size for nutritional survival by diffusion and neo-capillary ingrowth, i.e.,

TABLE 1

THE NUMBER, AND PERCENTAGE, OF SYNGENEIC EAR-IMPLANTED FETAL HEARTS SURVIVING AFTER FREEZE-THAWING, AS JUDGED BY MYOCARDIAL ELECTRICAL ACTIVITY BETWEEN 6TH AND 55TH DAYS AFTER IMPLANTATION. THESE INITIAL EXPERIMENTS SHOW COMPARISON BETWEEN FOUR SOLUTIONS USED FOR FREEZE PRESERVATION AND TWO METHODS OF THAWING

Immersion fluid	Time (hr) stored at −196° C	Thawing method	Electrical activity		
			6–15 Days	16–25 Days	>30 Days
A	72	MW[a]	16/27 59%	16/27 59%	12/27 44%
A	72	WB[b]	8/10 80%	7/10 70%	8/9[c] 89%
B	72	MW	7/7 100%	—	6/7 86%
B	216	WB	6/11 55%	—	6/11 55%
C	72	MW	0/5	0/5	0/5
C	72	WB	0/5	0/5	0/5
D	216	WB	0/6	0/6	0/4[c]

[a] Microwave thawing.
[b] Water bath thawing.
[c] Change in denominator: mice died.

the upper limit of size for survival without the need for direct vascular anastomosis at time of graft implantation.

DISCUSSION

In our search to establish possible methods for the preservation of mammalian organs in a deep frozen state, and their subsequent recovery, we have concluded that electromagnetic energy in a limited frequency range is essential for both the uniformity of thawing and the control of the thawing rate. From a consideration of the penetration depth of the wave, frequencies in the range from 500 to 5000 MHz (5 to 59×10^8 cycles/sec) are of possible interest; we have arbitrarily chosen a convenient frequency in the middle of this range, 2450 MHz. We have recently shown that microwave thawing at this frequency is an acceptable method for recovering tissue culture cells from liquid nitrogen storage (17) and that, in the case of canine kidneys, perfused with, for example, 10% (v/v) DMSO and then fluorocarbon, uniformity of heating from $-79°$ to $20° \pm 10°$ C is possible (13). The microwave insult is not inconsiderable in these cases: To achieve thawing rates above 100° C/min in an adult organ, the absorbed power density is of the order of 10 W/g. The electric field strength associated with the wave is correspondingly large, its actual value for any thawing rate depending on as yet unknown physical quantities, which we are also attempting to measure.

The electric field strength experienced by the thawed hearts in this case would be expected to vary from a peak value of 0 to a few thousand V/cm as the 1 mm organ in the 5 ml sample is rotated randomly through the resonant electromagnetic fields of the cavity, which is resonant in many (>20) modes. Only a very small fraction of this stored energy is absorbed in the heart and the solution in which it is frozen. The absorption of energy increases very rapidly above $-10°$ C; in a microwave cavity it is not possible to monitor the rate between, for example, $-60°$ and $-15°$ C (possibly the most critical range in freezing and thawing). Consequently, we established a mean rate (from $-196°$ to $+10°$ C) slightly above that possible in a water bath in order to make a comparison: The rates for the water bath and the microwave thawed heart should be about the same in the $-60°$ to $-15°$ C range. We intend subsequently to reduce the thawing rate (by changing the microwave power level) and use the system to investigate the relationship between thawing rate and different concentrations of different cryoprotective agents, and to monitor the absorbed power as the temperature changes.

The results reported in this paper show that the electrical activity of frozen fetal mouse hearts reoccur after microwave thawing. Statistically, with a limited number of hearts, there is no significant difference between microwave and water bath immersion. However, solution A and B are clearly significantly different from C and D and this is the basis for future work with this model.

SUMMARY

Hearts removed from 17–19 day fetal mice were frozen in liquid nitrogen and tested for electrical activity after rewarming. After exposure to various cryoprotective agents, hearts were cooled at 0.5–0.7° C/min. to $-100°$ C and then stored in liquid nitrogen for periods between 72 and 216 hr. Exposure to controlled microwaves at 2450 MHz or immersion in a water bath at 25 C was used in thawing. Electrical activity was studied for periods as long as 90 days after subcutaneous implantation into the ear of syngeneic adult mice. Overall, 59% of 54 frozen-thawed fetal hearts showed strong electrical activity after 30 days when the cryoprotective solution that had been used

contained 10% (v/v) dimethylsulfoxide (DMSO) and 10% (v/v) fetal calf serum in Hepes buffer. This system consists of a multicellular structure that is nourished by diffusion; it is well suited for the evaluation of different cryoprotective agents and for various thawing techniques.

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