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EMBRYO BANKS IN THE FUTURE OF DEVELOPMENTAL GENETICS

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THE initial discovery by POLGE, SMITH and PARKES (1949) that glycerol affords protection to spermatozoa during freezing and thawing subsequently led to the development of semen banks which have greatly facilitated livestock improvement especially in the cattle breeding industry. However, until recently, the few attempts to preserve mammalian embryos by freezing have been relatively unsuccessful (see reviews by AUSTIN 1961; HAFEZ 1969, 1971; WHITTINGHAM 1973a). The major advantage of preserving the mammalian embryo is that the embryo, unlike the spermatozoan or oocyte, contains the complete genome of the individual to which it will give rise and this can be propagated in a foster mother of unknown genetic background. The major drawback is the limited number of embryos that are available and therefore the freezing technique must have survival rates approaching 100% if it is to have any practical application. Such survival rates are extremely high when compared with those for spermatozoa frozen under optimal conditions (30–50% SHERMAN 1969). Nevertheless, the ability to preserve viable embryos at low temperatures has practical applications in genetics, developmental biology, and the animal breeding industry as well as contributing to the understanding of basic cryobiology. The purpose of this presentation is to outline a successful technique for the low temperature storage of embryos and to describe its application in the field of genetic research especially mouse genetics.

LOW TEMPERATURE STORAGE OF MOUSE EMBRYOS

Mouse embryos were the first mammalian embryos reported to survive freezing and thawing (WHITTINGHAM 1971a) but in this initial study they did not survive if kept frozen at −79° for longer than 30 minutes. A closer examination of the cryobiological factors which influence survival (suspending medium, cryoprotective agents, cooling rate, final storage temperature and warming rate) led to the development of an effective technique for freezing mouse embryos to −196° and −269° and resulted in high survival rates (50–70%) after storage at −196° for up to 8 days (WHITTINGHAM, LEIBO and MAZUR 1972). More recently, survival rates of up to 100% were obtained with embryos stored at −196° for up to 8 months (WHITTINGHAM and WHITTEN 1974).

The general procedures for recovering and manipulating embryos are ade-

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quately described elsewhere (BIGGERS, WHITTEN and WHITTINGHAM 1971; WHITTINGHAM 1971b).

1. *Source of mouse embryos:* All preimplantation stages of development (1-cell, 2-cell, 8-cell, morula and blastocyst) may be obtained from either naturally mated mice at various times after finding the vaginal plug (LEWIS and WRIGHT 1935; WHITTEN and DAGG 1961) or from superovulated mice at various times after the injection of HCG (EDWARDS and GATES 1959). The yield of embryos may be increased considerably by the latter treatment but the responses of different inbred strains to the gonadotrophins are variable; some strains are completely unresponsive. No difference has been detected in the survival of embryos obtained from superovulated and naturally mated mice after freezing and thawing although a contemporary comparison has yet to be made (WHITTINGHAM, unpublished observations).

All the preimplantation stages have been successfully frozen (WHITTINGHAM, LEIBO and MAZUR 1972; WILMUT 1972) but the 8-cell embryo is considered to be the most suitable stage for storage since it is easily obtained and its viability can be tested after thawing during a 24–36-hour culture period before transferring it to a suitably prepared foster mother.

2. *Media:* So far, the best medium for suspending mouse embryos for low temperature preservation is a modified Dulbecco's buffered medium—Table 1 (WHITTINGHAM and WALES 1969; WHITTINGHAM 1971a; WHITTINGHAM, LEIBO and MAZUR 1972). There is no decrease in viability of embryos stored in this medium at 0° for periods up to 48 hours (Table 2) and they are not affected by temperature shock when transferred directly to this medium at 0° (WHITTINGHAM 1972).

A modified Krebs-Ringer bicarbonate medium for mouse embryo culture is used for culturing the embryos recovered after freezing and thawing (WHITTINGHAM 1971b).

3. *Cryoprotective agents:* The most effective protective agent for freezing mouse embryos is dimethylsulphoxide (DMSO) (WHITTINGHAM, LEIBO and

TABLE 1

Composition of phosphate buffered medium (PBI) for mouse embryos storage

Component	mM	g/l
NaCl	136.87	8.00
KCl	2.68	0.20
$CaCl_2$	0.90	0.12
KH_2PO_4	1.47	0.20
$MgCl_2 \cdot 6H_2O$	0.49	0.10
Na_2HPO_4	8.09	1.15
Na.Pyruvate	0.33	0.036
Glucose	5.56	1.00
Bovine serum albumin (BSA)		3.00
Penicillin		100 U/ml
Dist. H_2O		up to 1 litre

TABLE 2

Comparison of viability of preimplantation embryos stored at 0° in PBS medium and modified Krebs-Ringer medium

Embryo stage	Medium	Percent embryos developing to blastocysts after storing at 0° for: (hr)						
		0	24	48	72	96	120	144
2-cell	PBS	83	78	46	33	35	18	0
	Krebs	85	43	18	3	0	0	0
8-cell	PBS	100	70	78	30	20	5	0
	Krebs	100	83	73	13	0	0	0
Morulae and	PBS	100	100	100	93	68	75	25
early blastocysts	Krebs	100	100	100	85	63	58	8

Values are mean percent of 4 experiments (15 embryos/replicate).
Source: WHITTINGHAM (1972).

MAZUR 1972; WILMUT 1972) but some protection is also afforded by glycerol (WHITTINGHAM, LEIBO and MAZUR 1972). Polyvinylpyrrolidone (PVP) afforded only limited protection to embryos frozen to −79° (WHITTINGHAM 1971a) and was completely unsuccessful in preserving embryos frozen to −196° (WHITTINGHAM, LEIBO and MAZUR 1972). Maximum survival of embryos was attained when they were suitably cooled in the presence of 1 to 2M DMSO. No damaging effects of the compound have so far been found.

4. *Freezing and thawing:* A major cause of freezing injury is intracellular ice formation; to prevent this, cells must be cooled slowly enough to allow all freezable water to flow out of the cell (MAZUR 1970). For cells similar in size to the mouse embryo (70μ in diameter) MAZUR (1963, 1966) has calculated that the cooling rate must be about 1°/minute or less. The optimum cooling rate for mouse embryos is between 0.3° and 0.8°/minute (WHITTINGHAM, LEIBO and MAZUR 1972). This value is consistent with the earlier theoretical calculations and is also one of the lowest cooling rates reported for animal cells.

The cooling procedures are based on those described by LEIBO *et al.* (1970). Briefly, the embryos are placed in 0.1 ml of phosphate buffered medium in 10 × 100 mm tubes, cooled to 0° and mixed with an equal volume of medium containing twice the desired final concentration of DMSO (usually 1M DMSO). The embryos are equilibrated with the additive for 15 minutes before transferring them to a seeding bath at −3.5° to −4.5°. Two minutes later, they are seeded and after a further 5 minutes are transferred to a 950 ml evacuated unsilvered Dewar flask containing 500 ml of continuously-stirred ethanol which is at approximately the same temperature as the seeding bath. The appropriate cooling rate is obtained by adjusting the degree to which this Dewar flask is immersed into an outer 4.3 litre evacuated and silvered Dewar flask filled with liquid nitrogen. The cooling rate is calculated from the time required for the samples to pass from −10° to −65°. When the samples have reached −80° they are transferred directly into liquid nitrogen.

The finding that mouse embryos were extremely sensitive to rapid thawing

(200°/minute or more) was a major discovery in the development of the mouse embryo freezing technique. Previously, slowly cooled cells, which presumably contain little or no intracellular ice, were shown to be relatively unaffected by the rate of warming (MERYMAN 1966). In contrast, rapidly cooled cells have to be warmed rapidly for optimum survival (600° to 1000°/minute) presumably to prevent damage caused by the recrystallisation of intracellular ice (MAZUR, LEIBO and CHU 1972). Optimum survival of mouse embryos is obtained with warming rates ranging between 4° and 25°/minute (WHITTINGHAM, LEIBO and MAZUR 1972; WILMUT 1972). These rates are achieved either by allowing the samples to warm in air (approximately 25°/minute) or by introducing the samples into a boiling tube (38 × 300 mm) containing 20 ml of ethanol precooled to −100° and allowing the tube to warm in air (approximately 4°/minute). This high sensitivity to rapid warming has not been reported for other animal cells but it may be necessary to prevent osmotic or structural damage during the hydration of these large embryonic cells (blastomeres). The suggestion that the zona pellucida which envelopes the preimplantation embryo is in some way responsible for the requirement of slow warming (WILMUT 1972) is unfounded since embryos which have had the zona pellucida removed show the same sensitivity to rapid warming (WHITTINGHAM, unpublished observations).

Immediately upon thawing, the DMSO is diluted out in a stepwise manner at 0°, to avoid possible osmotic shock, by the addition of 0.2, 0.2 and 0.4 ml of phosphate buffered medium at 45–60 second intervals. The contents of each freezing tube are emptied into an embryological watchglass containing 1 ml of medium and the tubes are rinsed with a further 1 ml of medium to ensure maximum recovery of embryos. After collection, the embryos are washed by transfer through 2 changes of fresh phosphate buffered medium (2 ml/wash) at room temperature.

5. *Assessment of viability of frozen-thawed embryos:* The criteria for assessing survival are those described previously (WHITTINGHAM 1971a), namely, the ability of embryos to develop to expanded blastocysts in culture and the ability to develop into living mice in the uterus of a foster mother. As mentioned earlier, the 8-cell embryo is considered the most convenient stage to store and these are usually cultured for 36–48 hours after thawing. The embryos developing to early blastocysts are then transferred to the uterus of suitably prepared pseudopregnant females (BIGGERS, MOORE and WHITTINGHAM 1965).

PRACTICAL APPLICATIONS OF THE STORAGE TECHNIQUE

The recent demonstration by WHITTINGHAM and WHITTEN (1974) that mouse embryos similar to other tissue cells (Meryman 1966) show no deterioration in viability when stored for prolonged periods at −196° establishes the feasibility of the mouse embryo storage technique. The formation of mouse embryo banks similar to semen and other cell banks provides a unique opportunity for the conservation of genetic material which might otherwise be lost. All inbred strains, mutations and special genetic combinations can be preserved which will be an invaluable asset for genetic research.

1. *Banking of inbred strains:* A preliminary survey of the viability of frozen-thawed embryos from different inbred strains showed that no strain was totally resistant to freezing and thawing (WHITTINGHAM 1972); in fact, the survival rates were extremely high (Table 3). It is now proposed that banks for all inbred strains should be set up in conjunction with the major mouse breeding centres concerned with the production of pedigree stock. For the first time in mammals, genetic pedigree standards can be established and this will enable a check to be made for genetic drift in subsequent generations.

The banking of inbred strains in various breeding centers will provide protection against hazards such as fire and disease. A colony can be quickly reestablished from frozen stocks. Transportation between countries with complex restrictions will be made easier (WHITTINGHAM and WHITTEN 1974). Finally, on purely economic grounds, inbred strains which are not in demand may be kept frozen providing space for the production of those strains in current use.

2. *Banking of mutations:* Many mutations that arise during the routine production of inbred strains of mice are destroyed because it is uneconomical to keep them when no immediate use is found for them. It would therefore be extremely advantageous to preserve them for future experimentation.

3. *Banking of recombinant inbred strains:* In recent years, many recombinant inbred strains of mice have been derived from various hybrid crosses and these have been used to identify and determine the linkage and function of histocompatibility and other genes (BAILEY 1971). Also, a variety of genes may be brought together in one animal for various experimental purposes and at the termination of the study the animals are destroyed. All these types of animals could be preserved for future use without great financial expenditure except for the cost of liquid nitrogen refrigeration.

4. *Banking of embryos for use in early developmental studies:* Banking of embryos could be used effectively for a number of experimental procedures in developmental biology, e.g., to obtain sufficient embryos for biochemical analyses, to hold embryos until sufficient numbers of different strain combinations are available for producing chimeras (TARKOWSKI 1961) or for preserving inner cell mass cells while obtaining sufficient blastocysts for cell transfer also to produce chimaeras (GARDNER 1968), and to hold embryos until suitable foster mothers are available for embryo transfer. Many more applications will accrue as the procedure is accepted into general use.

Precautions to be considered in the application of the storage technique: Until now, the main advantages of the low temperature preservation of mouse embryos have been discussed; however, there are certain precautions worthy of consideration since factors such as background ionizing radiation, the influence of the foster mother, and transmission of egg-borne viruses, may have detrimental effects upon the embryo during storage and/or subsequent development. So far, no adverse effect has been detected on the reproductive performance of mice derived from frozen embryos (WHITTINGHAM, LEIBO and MAZUR 1972; WHITTINGHAM 1972).

1. *Radiation sensitivity during storage:* At present, there is no information on

the effect of background ionizing radiation during the storage of mouse embryos, but all preimplantation stages have been subjected to varying levels of ionizing radiation *in vivo* and they appear to be most sensitive immediately after fertilization and at the morula stage (RUGH and WOHLFROMM 1962; RUSSELL and RUSSELL 1954).

Background ionizing radiation damage occurs during the long term storage of seeds when they are fully imbibed with water but the damage is reduced to a background level when they are dehydrated (ROBERTS 1972). If we assume that the mouse embryos are almost dehydrated during slow cooling then the effect of this type of radiation should be minimal; but considering the vast differences between plant and animal material an investigation of radiation on stored embryos is essential.

2. *Maternal influence on the developing fetus:* Several years ago MCLAREN and MICHIE (1958) demonstrated that the uterine environment of the mouse could have a specific effect on the skeletal development of the resulting fetus. This raises the question of whether the offspring derived from frozen-thawed inbred embryos will be identical to the parental strain or slightly modified by the uterine environment of the foster mother. The problem should be obviated by the use of foster mothers of identical or closely similar background. The claims for immunological adaptions originating from ova transfer are tenuous (UPHOFF 1972) but the ability to store frozen viable embryos will provide a more vigorous test of UPHOFF's hypothesis by permitting the simultaneous comparison of different generations of a substrain with the original ancestral strain (UPHOFF *et al.* 1973).

3. *Transmission of disease* via *the embryo:* There is indirect but reasonably conclusive evidence for the vertical transmission of murine lymphocytic choriomeningitis (LCM) virus via the oocyte (MIMM 1966) and murine leukaemia virus *via* the preimplantation embryo (FEKETE and OTIS 1954), although recent evidence indicates that the C type virus of murine leukaemia is carried *via* the genome (ROWE, HARTLEY and BREMNER 1972). Therefore, it would be impossible to prevent its transmission *via* the frozen embryo. Specific pathogen free (SPF) offspring could be obtained from frozen-thawed embryos recovered originally from SPC parents and transferred to SPF foster mothers after thawing.

Clearly, precautions can be taken to minimize the problems outlined above and they should not prevent the immediate application of embryo storage to the field of genetic research. It is anticipated that the technique will make as great a contribution to biological research as the banking of tissue culture cell lines and storage of semen has done in the last two decades.

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