STUDIES ON THE PRESERVATION OF MAMMALIAN EMBRYOS IN THE SUPERCOOLED STATE

by

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ABSTRACT

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Advances in the cryopreservation of bovine embryos have resulted in a current routine success rate of 64 % (in terms of fertility). However, in the case of bisected embryos, the success rate is less than 20 %. It was hoped that this loss of viability could be avoided by storage of embryos in the supercooled state, where the low temperature suppresses metabolism while circumventing ice formation and the osmotic stresses. Experiments were carried out with mouse embryos to identify optimal conditions for embryo preservation in the supercooled state.

Firstly, exposure of compacted morulae (CM) and early blastocysts (EB) to methanol (M) or glycerol (G) for 1 h at room temperature followed by culture in standard culture medium showed that the embryos tolerated up to 12 to 24% methanol or 24 to 48% glycerol. Next, the effects of stage of embryo (CM vs EB), preservation temperature (-2, -5, -10, -15° C) and concentration of 1:1 M:G (1 - 6% each) on embryo survival, assessed by culture after supercooled storage were tested. EB survived longer than CM under all conditions. Increased concentrations of cryoprotectants (M and G) increased the survival of supercooled embryos, but survival was decreased with the storage temperature. Replacing G with propanediol (P) significantly increased blastocyst survival at lower temperatures, but only at 6%M:6%P.

Exposure for 1 h to > 0.6 M of sucrose or trehalose at room temperature suppressed growth in culture, but dehydration in up to 0.4M sucrose, before supercooling (in M:P) increased survival at -5 or -10° C, survival increasing with dehydration.

Finally, demi-embryos (produced by destroying 1 or 2 blastomeres of 2- or 4-cell embryos, respectively) and intact embryos were cultured to the blastocyst stage,

stored at -5° for 48 h, then cultured for 24 h and transferred into pseudopregnant recipients. Supercooled storage did not impair the developmental potential of whole or demi-embryos *in vitro*, nor was there a difference between whole and demi-embryos with respect to growth *in vitro*. Similarly, there was no effect of supercooling on development of intact or demi embryos after transfer into pseudopregnant recipient mice, but demi-embryos developed more poorly than whole embryos($P < 0.05$). This was considered to be partly due to the lesser ability of demi-embryos to maintain luteal function and establish pregnancy.

These results indicate that the preservation of embryos in the supercooled state for short term storage is feasible. Moreover, by supercooling, even demi-embryos can be preserved at sub-zero temperatures with minimal loss of viability.

LE DEVELOPEMENT D'UN SYSTEM DE PROCEDE POUR LA PRESERVATION D'EMBRYONS MAMMIFERES DANS L'ETAT SUPERCOOLED

RESUME

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Les développements technologiques en cryopréservation d'embryons bovins ont permis d'en faire une procédure routinière, avec un taux de succès de 64% (par rapport à la fertilité). Par contre, lorsque l'on utilise les manipulations employées pour les embryons entiers dans la cryopréservation d'embryons bisectés, le taux de gestation se voit diminué à moins de 20%. Cette perte de viabilité peut être évitée en entreposant les embryons dans l'état supercooled, ou la basse température suprime l'activité métabolique, pendant que la formation de glace et la force osmotique liée au gel et dégel sont circonvenues. Trois séries d'expériences ont été entreprises, utilisant l'embryon de la souris comme model expérimental, pour identifier les conditions idéales pour la conservation des embryons dans l'état supercooled.

Dans la première série, deux expériences ont été excécutées afin de déterminer la toxicité du méthanol (M) et du glycérol (G), ainsi que d'identifier les conditions idéales pour maintenir la viabilité des embryons supercooled. Dans l'expérience 1.1, le morula compacte (CM), et le jeune blastocyste (JB), ont été exposés au méthanol ou au glycérol à la température ambiente pendant 1 heure. Le CM et le JB fûrent ensuite cultivés dans un milieu standard pour la culture d'embryon de souris, pour ainsi déterminer la toxicité des cryopréservatifs. La toxicité était apparante après une exposition de 12 à 24% de méthanol, ou de 24 à 48% de glycérine. L'expérience 1.2 examinait les critères suivants sur la

survie de l'embryon; le stage embryonnaire (CM vs JB), la température de préservation (-2, -5, -10, et -15° C) et la concentration de M:G à 1:1 (1-6% chaque). La survie des JB fut significativement plus élevée que celle des CM dans toutes les conditions examinées. En augmantant la concentration des cryopréservatifs, la survie des embryons supercooled (de 6h et 24h) était également augmentée. La survie embryonnaire a diminué lorsque l'on a abaissé la température d'attente. Dans l'expérience 1.3, le glycérol fut remplacé par le propanediol au même pourcentage (1-6%), et a demontré qu'à 6% de propanediol, la survie des blastocystes à basses températures était augmentée.

Dans la deuxième série d'expériences, les effets de la déshydratation et du supercooling d'embryons étaient étudiés dans un milieu contenant du sucrose ou du trehalose. Dans l'expérience 2.1, les JB ont été exposés à plusieures concentrations de sucrose ou de trehalose à température ambiente pendant une heure. Cette expérience avait pour but de déterminer le niveau de tolérance des JB à des agents déshydratants. Le stress osmotique était évident lorsque les agents étaient présents à des concentrations de 0.6M à 0.8 M. Dans l'expérience 2.2, les effets déshydratants du sucrose à différents niveaux et à des concentrations maximales de 0.5 M, ont été étudiés pour évaluer la survie des JB entreposés à -5 ou -10° C. La déshydratation à concentration maximale de 0.4 M de sucrose, a pu améliorer le taux de survie des JB, les plus hautes concentration de sucrose étant associées aux plus hauts taux de survie des embryons à -5 ou -10° C.

Dans la troisième série d'expériences, nous avons entrepris d'étudier la possibilité de développement in vitro et in vivo d'embryon entier ou demi après un entrepoage à -5° C à l'état supercooled pour 48 heures. Les embryons ont été cultivés après le supercooling pendant 48 heures et fûrent transférés dans des souris

pseudogestantes receveuses. Les demis embryons ont été générés en détruisant un blastomère d'un embryon contenant deux cellules, ou en gardant deux blastomères d'embryon contenant 4 cellules. L'entreposage de demis ou d'embryons entiers à l'état supercool, n'a pas endommagé leur potentiel de dévelopement. Aucune différence ne fut observée entre les 2 types d'embryons par rapport à leur croissance in vitro, cependant, après le transfert d'embryons dans la souris pseudogestante receveuse, il n'y avait aucun effet du au supercooling, mais les demis embryons se developpèrent plus difficilement ($P < 0.05$). Ce résultat peut être du au manque d'habileté des demis embryons à maintenir une fonction lutéolitique et à établir une gestation.

Les résultats obtenus indiquent que la préservation d'embryons à court terme est possible. De plus, les demis embryons peuvent également être conservés à des températures sous zero par le supercooling, ce qui permet une perte minimale de viabilité.

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INTRODUCTION

GENERAL

Commercial bovine embryo transfers were introduced in 1970, using at first, surgical procedures, by the world's first embryo transfer enterprise, Alberta Livestock Transplants (reorganized, in 1987, as AltaGenetics). During the late 1970's, nonsurgical methods were adopted and, in conjunction with superovulation, steady improvement in the recovery of embryos and in pregnancy rates following transfer, commercial transfers grew rapidly in popularity. A major stimulus to the growth of the industry was the then prevailing peculiar circumstance that, in the absence of a quarantine station in the United States, exotic or otherwise desirable cattle could be imported only from Canada, in the form of embryos transferred into and "shipped" in host cows. As the efficiency of transfers improved, costs gradually fell and the embryo transfer industry continues, to-day, to facilitate rapid improvement of farm herds by introduction of superior genetic material from commercial donors and by accelerating reproduction of superior stock.

The potential of embryo transfer was increased by success in bisecting bovine embryos (Willadsen et al, 1981; Wilmut et al., 1986) and the observation that the viability of demi-embryos is virtually the same as that of intact embryos (Willadsen 1979, Williams et al, 1984 and Baker et al, 1985). This finding has resulted in adoption of embryo bisection as a routine operation in many embryo transfer concerns, inasmuch as simultaneous transfer of both halves would thus double the probability of a pregnancy. An additional benefit is the possibility of twins, without the risk of producing a freemartin, as calves resulting from bisection are, perforce, of the same sex. Moreover, bisection of embryos, if used only to increase the number of embryos from a given donor, in effect, halves the cost of each embryo. Alternatively, one half embryo may be used to determine the sex of the other, by serological (Wachtel, 1984), cytogenetic (Hare et al., 1976) or DNA probe (Singh & Jones, 1982) techniques. Many of the potential benefits of routine bisection of embryos may be realized only if there is a possibility of storing one or both demi-embryos.

A natural consequence of the production of multiple embryos, often without an immediately available client, was the need for a means of storage of valuable embryos

pending sale thereof. This led to intensive research into cryopreservation, which offered an obvious solution. The feasibility of cryopreservation had already been demonstrated in the pioneering work of Whittingham, Leibo and Mazur (1972) with mouse embryos and subsequently applied to bovine embryos by Wilmut and Rowson(1973a). Cryopreservation was eagerly exploited in the burgeoning embryo transfer industry because of its immediate application in expediting the transport of embryos to remote clients, relieving the industry of the necessity of having synchronized recipients on hand and obviating the need to transport recipients themselves, with attendant high costs. Cryopreservation also permits storage of embryos for extensive periods without damage or genetic change and thus is important as a means of preserving valuable germplasm, particularly in the case of rare breeds and endangered species. Technological advances have increased the reliability of cryopreservation for bovine embryos, to the extent that cryopreserved embryos show a survival rate, following transfer, approaching that of transferred fresh embryos. Unfortunately bisected embryos survive cryopreservation poorly, with an expected pregnancy rate, after transfer, of only 20% or so. The reduced survival is thought to be due to the additive damage caused by both freezing and bisection. In freezing and thawing living cells, some damage appears to be inevitable. In the case of bisected embryos, however, the mechanical damage incurred during the splitting is added to the damage due to freezing and thawing. The resultant lower number of viable cells in the daughter embryos is thought to be inadequate to provide for all the embryonic structures necessary for normal development and, as well, to be less capable of generating a strong antiluteolytic signal (Heyman, 1985).

The principal sources of damage and the major causes of cell death are considered to be: a) the mechanical effects of intracellular formation of ice; b) solution effects, that is, damage caused by dehydration resulting in exposure of cells to increased solute concentrations, changes in pH and precipitation of solutes at their eutectic points (Lovelock, 1953); c) the "channel effect" due to extracellular ice formation, in which growing plates of ice gradually compress the cells trapped in the progressively shrinking channels of still unfrozen medium (Mazur et al., 1973; Schneider and Mazur, 1987). These effects are compounded by loss of membrane plasticity, which renders cells more vulnerable to deformation due to compression or

dehydration. Dehydration results when the tonicity of the external medium is increased by removal of water into ice, causing water to be drawn from the cell osmotically (Mazur 1970; see also Fiser and Fairfull, 1990). Dehydration has several consequences: Embryonic cells shrink as intracellular water flows into the extracellular space, leading to a rise in the cytoplasmic solute concentration. High concentrations of electrolytes resulting from the removal of cell water by exosmosis, or of medium water as ice, may modify the secondary and tertiary structure of macromolecules and extract lipids from membranes. Thus, the primary injury caused by solution effects may be a membrane lesion, the damage occurring when shrinkage and, consequently, electrolyte concentrations, reach critical levels (Lovelock, 1953; Meryman et al., 1977). Steponkus et al., (1985) suggested that during shrinkage cells lose so much plasma membrane that lysis occurs upon thawing. Regardless of the mechanisms of injury, embryos only survive slow freezing when protected by a permeating cryoprotectant.

As an alternative to freezing, storage of cells in the supercooled state appears to offer attractive advantages. Supercooling would provide the low temperature needed to suppress metabolic activity, but would eliminate the crystallization of water and the damage this inflicts, as well as the damage caused by osmotic changes during freezing and thawing. Although success in preserving cells in the supercooled state has been demonstrated with yeast cells (Mathias, et al. 1985) and turkey semen (Zavos and Graham, 1981, 1982), the approach has not been applied to mammalian embryos. We have attempted to develop a protocol for the storage of mouse embryos in the supercooled state, utilizing low temperature without freezing, to suspend metabolism while preserving viability.

LITERATURE REVIEW

Routinely successful deep-freezing of bull semen from the early 1950's and the understanding of cryobiological principles gained from the development of the requisite procedures led to attempts at freezing and storage of ova and embryos, mainly of laboratory mammals (Polge, 1980). However, the greater volume and correspondingly large water content of eggs and embryos presented a challenge which required 20 years to overcome (Sherman et al, 1958). Success in the cryopreservation of embryos was first achieved with the mouse in the pioneering work of Whittingham, Leibo, and Mazur (1972) in the United States and, independently, by Wilmut (1972) in Great Britain. The protocols developed by these workers have served as the basis for virtually all subsequent procedures for the cryopreservation of the embryos of all animal species.

Improvements in procedures for superovulation, handling and storage of embryos *in vitro* combined with an increasing understanding of basic cryobiological principles in the sixties and seventies (Mazur, 1961, 1972 and 1977) resulted in development of deep-freezing methods for mouse embryos (Whittingham, Leibo and Mazur, 1972, Wilmut 1972). During the succeeding years of the 70's, studies on the low temperature preservation of farm animal embryos were conducted, necessarily, on a smaller scale than those involving laboratory animal embryos, because of the difficulty of obtaining large numbers of livestock embryos for experimentation. Moreover, it was demonstrated that compacted morulae and blastocysts from cattle, treated the same way, also survived deep-freezing and 6% developed to term after transfer to appropriately synchronized recipients (Wilmut and Rowson, 1973b). However, the overall survival rate (6%) was too low to allow the technique any immediate importance in practice. The first deep-freezing experiments with sheep morulae and blastocysts (Willadsen et al, 1976a) were also based on the early procedures established with mouse embryos. Gradual improvements in procedures for the cryopreservation of bovine embryos in the intervening years have now made success rates of 64 % routine, which compares very favourably with the 72% pregnancy rate obtained by transfer of fresh embryos by the best embryo transfer practitioners (Lehn-

Jensen, 1986). Cryopreservation has now been incorporated into most commercial embryo transfer operations as a routine option.

The principal steps in mammalian embryo cryopreservation, as applied in various laboratories (Whittingham et al.,1972, Bielanski et al., 1986), may be summarized as follows:

1. Gradual introduction of the cryopreservative,
   i.e. equilibration, allowing time for the cryoprotectant
   to slowly permeate the cells of the embryo;
2. Cooling to temperatures just slightly below the freezing
   point of the suspending medium;
3. Inducing ice crystal formation by seeding or vibration;
4. Slow cooling to between -30 and 40° C to accommodate slow
   exosmosis of intracellular water, i.e. osmotic dehydration;
5. Storage in liquid nitrogen;
6. Rapid thawing and gradual removal of the cryopreservative
   from the embryos.

It should be noted, however, that the optimal rewarming velocity depends on the final temperature reached in the slow cooling phase. Some other current techniques use slow cooling to about -80°. In such cases, thawing must be slow (Massip et al., 1989).

Alternate procedures

Recent work has focused on procedures that would eliminate the need for controlled freezing and permit embryos to be cryopreserved by direct transfer into liquid nitrogen. These approaches reduce the time needed for cooling and warming and eliminate the need to induce extracellular crystallization (seeding). Alternative cryopreservation procedures can be divided into three categories, based on the extent of dehydration prior to cooling and on whether extracellular crystallization occurs during cooling or warming (Rall, 1987). In the first approach, embryos are partially dehydrated prior to cooling by exposure to a mildly hypertonic solution of sucrose and a permeating cryoprotectant (only the cryoprotectant enters the cells) before freezing, either by the two-step method (Walter et al., 1975; Farrant et al.,1977) or by using a moderately rapid (20 to 30° C/min) cooling rate (Miyamoto et al., 1983; Williams, et

al. 1986).

A second approach, ultrarapid freezing, involves more extensive osmotic shrinkage, exposing the embryos to a more hypertonic solution containing sucrose (ca. 0.5 M) and high concentrations of glycerol (3 to 4 M) (Szell et al., 1986). The glycerol is allowed to permeate into the shrunken embryos and then the embryo suspension is frozen rapidly (> 2500 °C/min) in liquid N_2. Although ice forms in the suspension during cooling and rapid warming, the embryos are sufficiently dehydrated prior to cooling to avoid intracellular crystallization (Rall, 1987; Rall et al., 1987). In extreme cases, as when embryos are slowly cooled to -70°, extensive dehydration occurs as a result of gradual > 10x concentration of the medium.

A third technique, vitrification, represents a new approach, based on the fact that very concentrated solutions of cryoprotectants do not crystallize when cooled, but become increasingly viscous, eventually achieving a glass-like state (Rall and Fahy, 1985). Eventually the cytoplasm becomes so permeated with cryoprotectant as to remain unfrozen, but at temperatures well below the normal freezing point of the cytoplasm temperature. The transformation into a glassy state (vitrification), when very low temperatures are reached, represents an extreme form of supercooling (Rall, 1985a,b).

Cells are more freely permeable to water than to the protectants, so that at thawing, when the suspending cryoprotectant medium is diluted, water enters more rapidly than the cryoprotectant leaves and the blastomeres swell. Two methods are widely used to dilute the cryoprotectant at thawing in order to control the degree of swelling. The first is stepwise reduction of the concentration of the additive in a series of equilibrations at 0°C or 20°C with successively lower concentrations of the cryoprotectant (Whittingham et al., 1972, Wilmut, 1972). The second method is to transfer embryos into a medium containing a hypertonic concentration of a non-penetrating solute, such as sucrose, to counteract osmotic swelling (Leibo and Mazur, 1978). After the efflux of the additive, the embryos are returned to an isotonic solution. A sucrose dilution procedure was developed for bovine embryos within a plastic inseminating straw (Leibo, 1984, Renard et al., 1983).

Role of free and bound water in the cell

Water constitutes some 80 % of the total mass of most mammalian cells, thus an understanding of the water relations of the living cell is of great interest to biology in general, and to cryobiology in particular. Indeed, the movement of water across cell membranes during freezing and thawing plays a crucial role in determining whether the cell survives. Such movements involve only free water. However, a portion of the cell water exists in an immobile form, that is, as bound water.

Electron microscopy has revealed that a varying portion of the cytoplasm of mammalian cells is filled with various membranous structures or organelles including the endoplasmic reticulum and Golgi vesicles, which provide a framework for ordered arrays of active functional units essential to life and divide the cell into compartments in which water exists in free and bound forms.

Biological macromolecules, such as those which form these membrane structures, are associated in their native state, with a certain amount of bound water, due to the presence of charged groups which tend to form hydrogen bonds with water molecules. The presence of such water is a fundamental necessity for the maintenance of the native structure and preservation of function of the macromolecules, even during freezing (Kuntz and Kauzmann, 1974). Hydrogen bonding is so strong that water molecules are prevented from diffusing to the surface of growing ice crystals during cooling and, thus, from participating in the crystallization process.

Two-factor hypothesis of freezing injury

In the terminology of thermodynamics, freezing is referred to as a first order phase change, the melting point being the only temperature at which the solid and the liquid can coexist in stable equilibrium at a given pressure. The physical and physiological responses of embryos to freezing, like those of other mammalian cells, are responses to the combined effects of lowered temperature, loss of cell water and changes in the state of water. Freezing can preserve the viability of cells only when the cells are protected by the presence of suitable, protective agents. According to the two factor hypothesis of freezing injury (Mazur, Leibo and Chu, 1972), injury to a cell during freezing and thawing results from the interaction of two factors, **the formation of intracellular ice**, with attendant recrystallization during slow warming, and **solution effects**, as described above, in the introduction. Although both factors will be present, which of the two predominates in a particular case depends on the cooling velocity used. In rapidly cooled cells, the probability of intracellular ice formation is great because freezing can occur internally before free water can leave the cell and freeze externally (Leibo, et al., 1970; Mazur, 1970 and 1977). The use of slow cooling or of the two-step freezing procedures allows a degree of dehydration to occur before the temperature falls far enough to precipitate sudden intracellular freezing. Results obtained in applications of the two-step freezing procedure support the theory that cell survival is dependent on the proportion of intracellular ice (Walter et al., 1975; Farrant et al.,1977).

Although a small amount of intracellular ice is not inevitably lethal (Shimada et al., 1975; Bank et al., 1974), its presence produces a metastable state that may lead to injury, depending upon conditions during rewarming (Leibo, 1989). The formation of intracellular ice is compatible with cell survival, provided that the crystals are small and thawing is sufficiently rapid (Farrant et al., 1977; Fiser et al., 1984 and 1986). Internal crystal formation may be limited by using a sufficiently low cooling velocity, $\leq$ 1°C/min, which permits the exit of cell water so that the bulk of the ice formation takes place extracellularly.

On the other hand, when cells are cooled slowly, they may be exposed to potentially damaging, increased concentrations of solutes, both externally (due to ice formation) as well as internally (due to exosmosis). These alterations are examples

of the solution effects already described.

Cryoprotectants

Cryoprotective compounds found to protect mammalian cells against freezing damage generally fall into two groups (Doebbler 1966, Farrant et al. 1980): permeating solutes of low molecular weight and non-permeating solutes of high or low molecular weight.

Most permeating solutes, such as glycerol, DMSO, alcohols and various glycols have hydrogen-bonding substituents (OH, NH_2, =O) and bind strongly with water, lowering the freezing point of the solution. Properties which make them suitable for use in biological systems are low toxicity, ability to pass through cell membranes freely and the ability to dissolve electrolytes. The concentration of hydrogen-bonding substituents (providing high solubility in water) is well correlated to cryoprotective activity (Doebler et al, 1965). Their depression of the freezing point of the external medium, by reducing removal of water from solution into ice, also has the effect of reducing the exposure of the cell to elevated salt and solute concentrations (Meryman, et al., 1977).

Non-permeating cryoprotectants, such as sucrose or trehalose do not enter the cell, but are considered to exert a significant cryoprotective effect by causing osmotic dehydration of the cell. It is speculated that high molecular weight solutes such as dextran, serum and polyvinyl pyrrolidine (PVP) exert a cryoprotective action by covering cell membrane defects that may arise in the course of the cryopreservation procedures and/or by helping to repair damaged cell membranes during or after thawing (Williams 1983, Grill et al,1980).

Induction of ice crystallization; freezing, storage and thawing procedures

Whittingham (1977) found that when mouse embryos were frozen slowly, if significant supercooling, e.g. to 4° below the freezing point of the cells, was allowed to occur before inducing ice crystallization, sudden freezing of the supercooled system caused significant reduction in the viability of the embryos; no embryos survived sudden freezing after more extensive supercooling. It has become, therefore, general practice in embryo freezing procedures since the first success by Whittingham et al

(1972) to induce ice crystallization by "seeding" when the temperature of the sample falls slightly below its freezing point. This may be done by adding a small ice crystal to the supercooled solution, touching the side of the container with a metal rod cooled in liquid nitrogen, shaking or tapping the sample vessel, or by automatic seeding in a programable freezer (Christmas, 1983). After the phase transition, the freezing of a sample may be continued by either of two methods. The first is the two-step freezing procedure, described above. Walter (1975) and Farrant (1977) found that cells supercooled in media containing 1-2M DMSO or glycerol generally do not crystallize intracellularly at temperatures above -40° C. Therefore, after seeding, cells can be cooled rapidly to about -30° C and then, after a period of equilibration, plunged into liquid nitrogen. This approach is attractive because it requires only simple equipment. Mouse and bovine embryos have been frozen with good results using the two-step method (Kasai et al., 1980 and 1981; Wood et al.,1980).

The second approach to freezing after seeding is linear freezing, in which the temperature in the freezing chamber is gradually lowered to the temperature at which the sample may be transferred directly into liquid nitrogen. This method has been used extensively in the cryopreservation of embryos of the domestic species (sheep, cattle and horse).

No damage of the sort associated with processes of freezing and thawing takes place during storage in liquid nitrogen at -196° C. Studies on frozen 8-cell mouse embryos have shown that the effects of background radiation in amounts equivalent to the cumulative effects of 2000 years of storage are negligible and therefore normal background radiation would not be a hazard. (Glenister et al, 1984).

Thawing is also a phase of cryopreservation requiring particular care. Considerable damage can be inflicted on cells during thawing unless the optimal rewarming procedure associated with the particular cooling method used is chosen. The degree of dehydration achieved at the completion of cooling, as well as the permeability of the cell to water and to the cryoprotectants, are factors whose interactions determine the optimal rate of rewarming. The volume and surface to volume ratio of the cell are also important factors. Thus, when cooling has involved extensive dehydration (e.g. after slow cooling to -80°), then thawing must allow for rehydration. The optimal thawing procedure, in this case, must be slow enough to

avoid too rapid dilution of the medium by the melting of extracellular ice, causing a rapid influx of water into the cells and swift change in volume, which would exert excessive stress on cell membranes. On the other hand, when dehydration has been only moderate, rewarming must be sufficiently rapid (a relative term; the optimal rate also depends on the particular cell or tissue) to prevent regrowth or enlargement of intracellular ice crystals.

Supercooled storage

In conventional cryopreservation procedures involving slow freezing, embryos are exposed to solutions which gradually become more and more concentrated. This has a number of consequences for the cell, including dehydration and extraction of membrane constituents resulting in volume changes and membrane stresses as well as possible protein precipitation and/or denaturation due to pH shifts, increased ionic strength and reduced solubility due to cold (Lovelock, 1953). It is believed that preservation of cells at low temperatures, under conditions in which freezing of the medium is avoided (storage in a supercooled state or supercooled storage) would eliminate or mitigate the stresses due to the osmotic changes resulting from concentration of the medium by freezing. The new approach of supercooled storage exploits low temperature to inhibit metabolic activity in the cell, while circumventing the principal physical and chemical stresses associated with freezing and thawing (Zavos et al, 1981; Mathias et al, 1985).

The first indication of the potential of storage in the supercooled state was the report by Mazur (1961) that yeast cells can survive limited exposure to temperatures below –16°C, provided that the medium does not freeze. More recently, Mathias et al. (1985) found that viability can be preserved in yeast cells at –20° C for up to 16 weeks by storage in the supercooled state. These workers also found that plant cells can be supercooled to below –20° C and erythrocytes to below –35° C before ice forms within the cells. Supercooling has also been found to be a promising means of preserving turkey sperm which, after conventional freezing and thawing, show poor fertility (Sexton, 1980). Zavos and Graham (1981, 1982) reported that supercooled storage of turkey sperm at –20° C for 24 h, allowed maintenance of relatively good fertility.

Although there have been several attempts to preserve embryos at low

temperatures without freezing, these involved storage at 0° or 4° and limited survival to no more than a few days (Trounson et al., 1976; Hahn et al., 1978; Willadsen et al., 1978). Supercooled storage would be a logical extension to these attempts, using sub-zero preservation temperatures for more effective suspension of metabolic activity, without freezing. Supercooling methods have not yet been applied to the storage of embryos.

Nucleation events and avoidance in the supercooled state

Franks (1981), in an extended discussion of the biophysics of freezing, emphasized that water does not freeze spontaneously at the equilibrium freezing point because any crystallization process must follow a nucleation event. This involves the formation of an "embryonic" crystal, a configuration of critical groups of water molecules serving as a focus for the condensation of other water molecules with a reduction in their chemical potential. These nuclei can form spontaneously as a result of random fluctuations of density and energy within a liquid, or may be induced by any particle which acts as a "seed". Once a nucleus grows, by accretion of molecules, to a critical size, then crystallization takes place rapidly, at least at fairly low subzero temperatures. Near the melting point, the number of molecules required to promote clustering is very large, but the lower the temperature, the smaller the number of water molecules needed to form a critical nucleus. Therefore, the lower the temperature, the greater chance of spontaneous nucleation.

For the reasons summarized above from Franks' review, in order to prevent crystallization in media to be used for supercooled storage, the supercooled state has to be stabilized by the addition of agents which interfere with nucleation. Glycoproteins and other macromolecules act as antifreezes by "masking" nucleation foci. On the other hand, low molecular weight substances containing hydroxyl groups, e.g. sugars and glycols, bind water via hydrogen bonds and form a lattice which stabilizes the solution (Douzou, 1977).

OBJECTIVES OF THE RESEARCH

In order to eliminate damage caused by freezing and thawing, we have attempted to store embryos in the supercooled state, in which the low temperature suppresses metabolic activity but ice formation and osmotic stress due to freezing and thawing are avoided. Three series of experiments were carried out with compacted mouse morulae and/or early blastocysts to:

1) assess the effects of storage temperature and penetrating cryoprotectant concentration on embryo survival and determine the most suitable combination of solutes and temperature for maintenance of viability of the embryos supercooled in isotonic solution;

2) investigate the effects of dehydration on embryo survival in supercooled storage;

3) assess the effect of supercooled storage on the developmental potential of whole and half embryos, by culture *in vitro* and by transfer to pseudopregnant recipients.

MATERIALS AND METHODS

Embryos.

Female albino ICR mice used in three series of experiments were housed and treated according to standards of Canadian Council on Animal Care and all procedures were reviewed and approved by the Institutional Animal Care Commitee in Agriculture Canada. 3–5 weeks old were induced to superovulate by intraperitoneal injections of 5 IU pregnant mare's serum gonadotropin (PMSG, Equinex, Ayerst Laboratories, Montreal, Quebec) followed, after 48 h, by 5 IU of human chorionic gonadotropin (hCG, chorionic gonadotropin, Sigma Chemicals, St. Louis, MO). The mice were placed overnight with ICR males and examined the following morning for the presence of vaginal plugs. Mice were killed by cervical dislocation, as required, to allow embryo recovery. Embryos at the 2– to 4–cell stage, compacted morulae (CM) and early blastocysts (EB, blastocoel volume less than 50% of embryo) were collected 55, 78 and 90 h, respectively, after hCG administration, by flushing the excised oviducts (2– to 4–cell embryos) or uterine horns (CM and EB) with modified phosphate–buffered saline (M2, a modification of a standard culture medium, containing the non–toxic buffer, HEPES, as a replacement for most of the usual sodium bicarbonate, permitting manipulations in an atmosphere of air without change in pH. See Fulton and Whittingham, 1978; Quinn et al, 1982; the composition is given in Appendix A.). After washing the embryos in M2, morphologically normal CM and/or EB were selected for experimental use. All manipulations of embryos prior to cooling were conducted at room temperature (25° C).

Media.

Basic supercooled storage medium (BM). Except as noted, all reagents were obtained from Sigma Chemicals.

Permeating cryoprotectants, in the amounts required (see descriptions of specific experiments, below and in Appendix A) were added to a synthetic medium (developed by Dr. P.S. Fiser at the Animal Research Centre of Agriculture Canada),

containing bovine serum albumin (BSA), dextran, glucose, glycine, proline, ribose, sorbitol, trehalose, tris hydroxymethyl aminomethane (TRIS buffer) and N-tris (hydroxymethyl) methylaminosulphonic acid (TES buffer). The pH of the final medium was adjusted to 7.0 and the osmolality, nominally 330 mOsmols/kg, was checked (a value within ± 10 mOsmoles was acceptable), using a Wescor Model 5500 vapour pressure osmometer (Wescor Inc., Logan, Utah). The medium was designed to incorporate glycine, proline and trehalose for their reported ability to preserve membrane structure and function during cooling (Chu, 1978; Rudolph and Crowe, 1985), dextran and BSA for their ability to repair damaged cell membranes (Williams, 1983; Grill et al., 1980) as well as to suppress nucleation (Douzou, 1977).

Culture medium. Whittingham's mouse embryo culture medium, M16 (Whittingham, 1971; see Appendix A) was used for routine culture. M2 is a modification of M16 incorporating the non-volatile buffer HEPES to permit manipulation of embryos in an atmosphere of air.

Statistical treatments. Results were subjected to χ^2 analysis and to ANOVA using the SAS computer program (SAS Institute Corp. Cary, North Carolina) on a Digital Equipment VAX computer (see Appendix C)

EXPERIMENTAL PROTOCOLS

SERIES I

Experiment 1.1: Tolerance to the cryoprotectants. The tolerance of mouse embryos to methanol and glycerol, individually, was assessed by exposing CM and EB for 60 minutes at 25° C to BM containing 6 to 48% (v/v) of methanol (Analar grade, BDH, Toronto, Ont) or 3-48% (v/v) of glycerol. After the exposure, the embryos were washed 3X in M2 medium, then in M16 and cultured under silicone oil (dimethylpolysiloxane, DMPS--2X, Sigma) in 20 μl droplets of M16, using 35 mm diameter plastic culture dishes (Falcon), in groups of 10 embryos per droplet and 16 droplets per dish, in an atmosphere of 5% CO_2 in air at 37° C. Survival was determined as the proportion of embryos which formed expanded blastocysts during

48 h of culture. At least 20 embryos were used in each treatment. The design of the experiment is shown diagrammatically in Fig. 1.

Experiment 1.2: Supercooling of embryos. Preliminary observations indicated that the deleterious effects of exposure to methanol and glycerol were not additive when both were present. In supercooling experiments, solutions with a combined concentration below the highest concentrations tolerated individually were used. The solutions for assessing supercooled storage were BM containing 1%, 3%, 6%, each, of methanol and glycerol. These media were designated BM.MG11, BM.MG33 and BM.MG66, respectively.

The effects of stage of embryo development, preservation temperature, test medium and storage time were evaluated as follows. Compacted morulae or early blastocysts, in groups of 10 to 15, were equilibrated with the supercooling medium for 20 min. then loaded into a 0.25 ml plastic straw (I.M.V. Société Anonyme, L'Aigle, France) by aspiration. The droplet of medium containing the embryos was separated from additional droplets of medium by small air spaces. The straw was sealed by plastic caps and placed horizontally in a cold chamber, set at the required storage temperature (ATC-40, FTS Systems Inc, Stone Ridge, N.Y.) and monitored by a thermocouple probe connected to an outer thermometer (Omega450 ATT, thermocouple thermometer type T) and allowed to cool to the required storage temperature (–2 to –15° C). The observed cooling rate was approximately 1° per minute. After storage for 2-24 h, the straw was warmed rapidly by immersion in water at 25° C. The embryos were expelled into a watch glass by cutting the straw, placed in BM for 15 min, washed 3X in M16 and cultured to assess survival, as in the previous experiment. The design of the experiment is shown diagrammatically in Fig. 2.

Experiment 1.3: Propanediol as a cryoprotectant. Because of reports of the effectiveness of propanediol as a cryoprotectant in freeze preservation of mouse (Ko et al., 1988), hamster oocytes (Hyatt Sachs et al., 1989), rabbit embryos (Renard et al., 1984) and human embryos (Testart et al., 1986 and 1987), the effect of replacing glycerol in the media used for supercooling with the same percent (v/v) of propanediol

was tested. The media containing propanediol are designated BM.MPnn where n is the percent of methanol or propanediol. Survival after supercooled storage was determined as in the previous experiments.

SERIES II

Experiment 2.1: Effects of partial dehydration in the presence of cryoprotectants. The effect of exposure to dehydrating concentrations of sucrose and trehalose in the presence of methanol and propanediol was tested by assessing the survival of embryos exposed to the individual cryoprotectants for 60 min at 25° C in BM.MP33 containing 0 to 1.0 M of sucrose or of trehalose. EB were then suspended in BM.MP33 for 10 min. Half were then exposed to BM.MP33 containing 0 to 1.0 M sucrose or trehalose for 60 min at 25° C in a watch glass. The remaining embryos were placed in BM.MP66 for 10 min, then transferred into BM.MP66 containing sucrose or trehalose for 60 min. After exposure to the dehydrating medium, the embryos were washed, first in BM for 10 min, then 3X in M16 and cultured for 48 h as described above (Series I). Survival of the embryos as before, was determined as the proportion which developed into expanded blastocysts during 48 h of culture *in vitro*. A control group of embryos(10–15) was cultured in each trial without any treatment to check on intact embryo viability in *in vitro* culture conditions. At least 20 embryos were used for each treatment. Figure 3 summarizes these procedures.

Experiment 2.2: Supercooling dehydrated embryos. To investigate the effects of storage temperature (–5, –10° C) and degree of dehydration, i.e. sucrose concentration (0 to 0.5M), EB, 10 –15 per group, were equilibrated with BM.MP33 for 10 min at 25° C in a watch glass. Half the embryos were then transferred into BM.33 containing 0 to 0.5M sucrose, equilibrated for 8 min at 25° C, loaded into 0.25 ml straws and then cooled to the required holding temperature, as described above for Series I, Experiment 2. The remaining embryos were incubated for another 10 min in BM.MP66, transferred into BM.MP66 containing sucrose, equilibrated for 8 min at 25° C and cooled to the required holding temperature. After storage for 24–72 h, the straws were warmed rapidly by immersion in water at 25° C. The embryos were expelled into watch glasses and equilibrated, for 10 min each, in reverse order, in the same media used before transfer into the supercooling media. The embryos were then

washed 3x in M16 and cultured, as in the previous experiments, to assess survival. The experimental protocols are summarized in Figure 4.

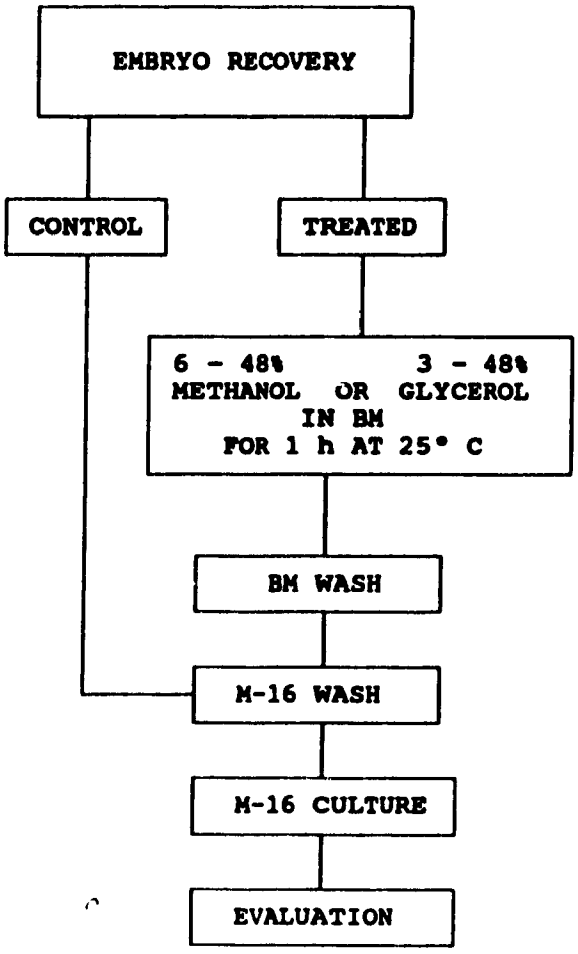
SERIES III

Production of demi-embryos and supercooled storage. Demi-embryos were prepared from normal 2- or 4-cell embryos by microsurgery. With the aid of an inverted microscope providing phase-contrast illumination, (magnification 100x), and fitted with Leitz micromanipulators, the embryos were immobilized with a holding pipet in a 50 μl droplet of medium M2. Several such droplets were arranged in a 60 x 15 mm plastic culture dish (Falcon) and covered with light paraffin oil (Fisher Chemicals, Fisher Scientific, Nepean, ON). Half the blastomeres of each embryo were destroyed by puncturing them with a fine glass needle. Demi- or whole embryos were cultivated to the blastocyst stage in 20 μl droplets of M16 described previously. Blastocysts obtained in this manner were supercooled in BM.MP33 containing 0.3 M sucrose 48 h at -5° C, using the protocol shown for BM.MP33 in Figure 4. After storage for 48 h, the straws were warmed rapidly by immersion in water at 25°C. After culture *in vitro* for 24 h, using the culture procedure described previously, embryos which showed normal development were selected and transferred into pseudopregnant recipients, as described below.

Survival *in vivo*. Females of the PC strain, developed from several inbred strains (C3H/He, C57BL/6J, CBA/J and SWR/J) at the Animal Research Centre, Agriculture Canada (Nagai & Kristjanson, 1970), were used as recipients. They were caged with vasectomized ICR males proved to be sterile and checked every morning for the presence of a vaginal plug. The day on which the plug was found, was designated as Day 1 of pseudopregnancy. Recipients were maintained under conditions of 12 h light: 12 h dark periods, 40-50% humidity at 20-24 °C of temperature.

To examine the developmental potential *in vivo* of the demi- and intact treated or control (blastocysts which had not been supercooled), 4 to 8 embryos were transferred surgically into each uterine horn of recipients on Day 3 of pseudopregnancy, using the method of Hogan et al. (1986). Development was assessed by counting fetuses at autopsy on Day 15 or 16 of pregnancy.

Figure 1: Test of tolerance to methanol and glycerol.



M-2 Medium, at 25° C.

CM: 78 h post HCG. Flush both oviduct and uterus.
EB: 90 h post HCG. Flush uterus. Recover < 50% with blastocoel

3X at 25° C.

3x at 25° C.

48 h, 5% CO_2 in air, 37° C.

Figure 2: Supercooling of embryos.

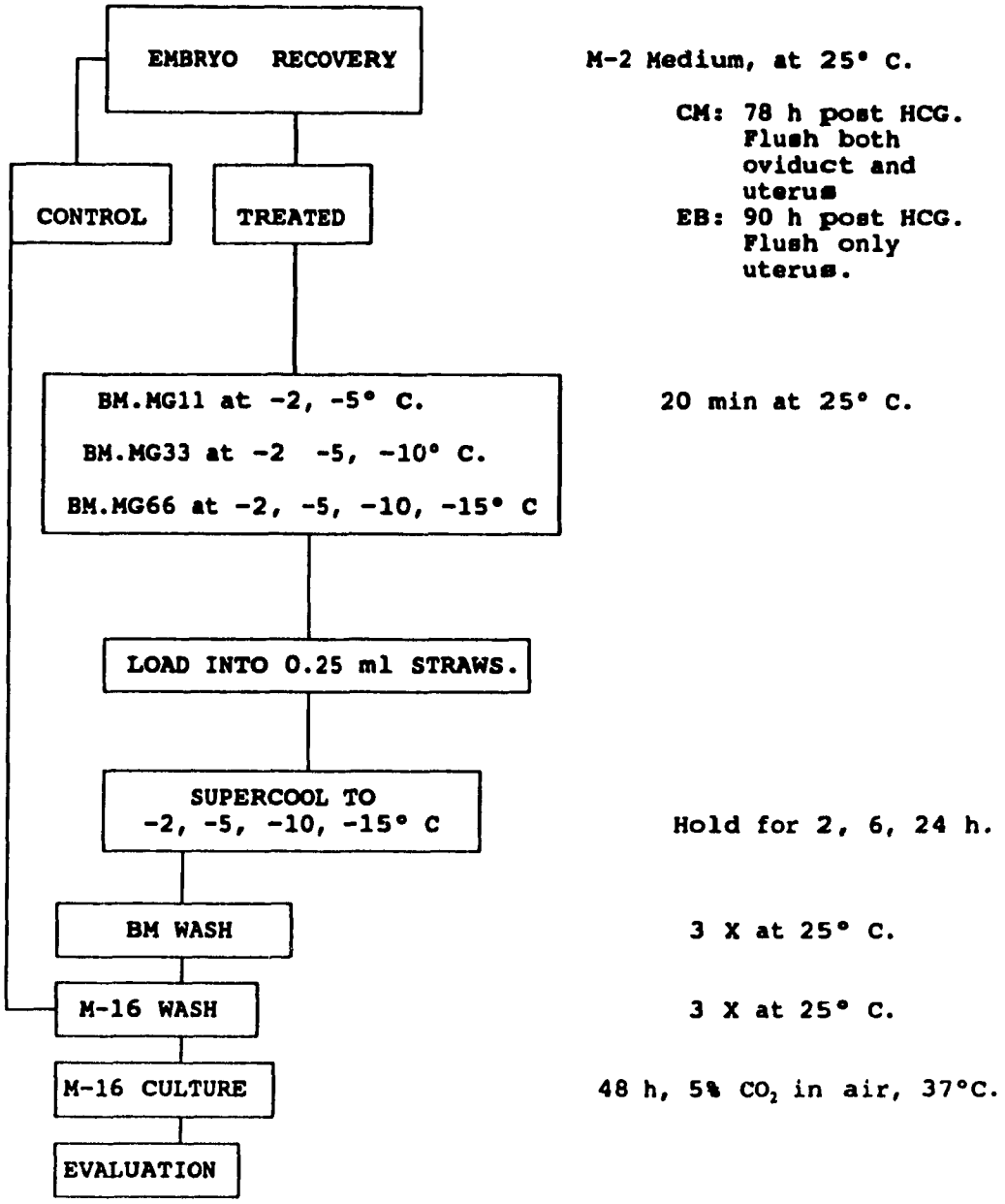


Figure 3: Effects of partial dehydration in the presence of cryoprotectants.

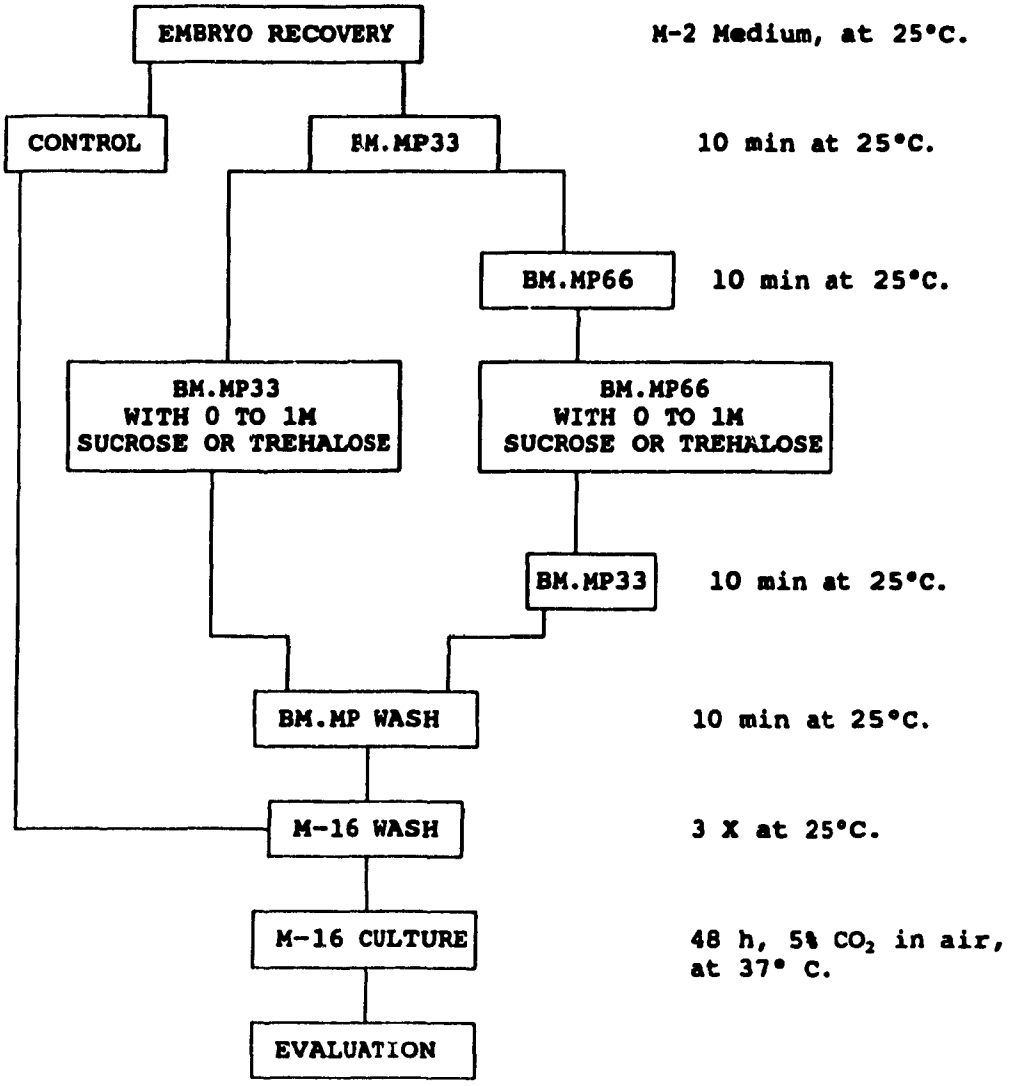


Figure 4: Supercooling dehydrated embryos.

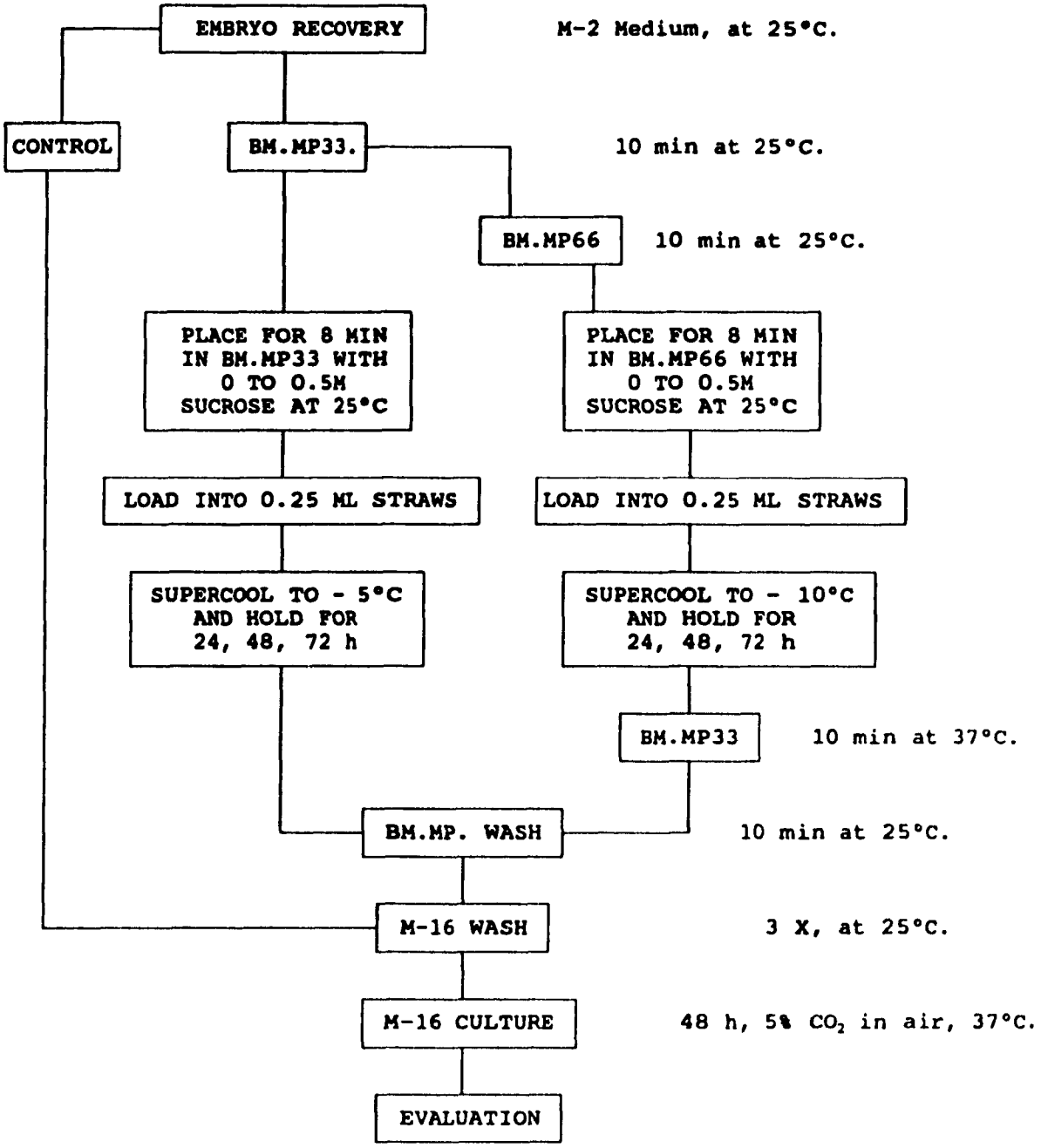
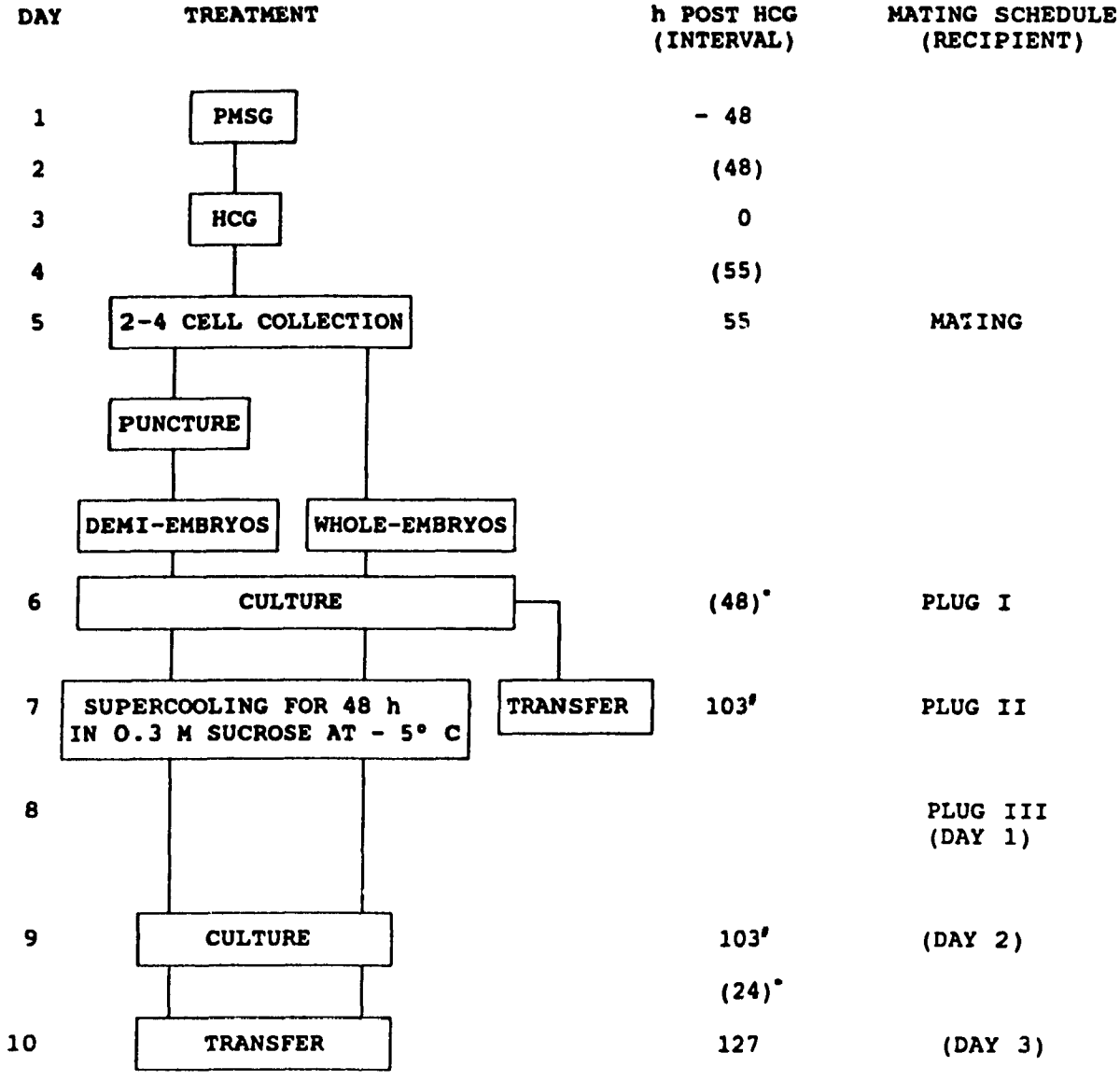


Figure 5: Assessment of developmental potential of supercooled embryos *in vitro and in vivo.*



*Total *in vitro* culture time is 48 + 24 = 72 h.
#During supercooling, it is expected that the development would be stopped temporarily. Therefore, the time may be the same as pre- supercooling.

RESULTS

Series I

Experiment 1.1: Tolerance to the cryoprotectants.

The proportions of embryos developing to expanded blastocyst after exposure to various concentrations of permeating cryoprotectant agents are presented in Table 1 and 2. Exposure to glycerol for 1 h was well tolerated by both CM and EB at concentrations up to 24%, regardless of embryonic stage. Methanol was less tolerated by the embryos than glycerol: no embryos survived exposure to 24% methanol, whereas >90% survived exposure to 12% methanol or to 24% glycerol (the molarity of 12 % methanol >> that of 12 % glycerol).

Experiment 1.2: Supercooling of embryos.

Tables 3 and 4 show the effects of different concentrations of glycerol-methanol and storage temperature on the survival of compact morulae and early blastocysts, respectively. The observations are also illustrated 3-dimensionally, for each cryoprotectant concentration, in Figures 6 to 8. The media with the least amounts of cryoprotectant BM.MG11 and BM.MG33 froze when cooled to -10° C and -15° C, respectively. Consequently, no embryos survived those combinations of medium and temperature. There was significant interaction between the effects of cryoprotectant concentration and storage temperature. Survival in supercooled storage increased with the concentration of the cryoprotectant ($P < 0.05$), but decreased with the storage temperature ($P < 0.01$). EB withstood longer storage than CM at any storage temperature tested ($P < .001$). The survival of CM after storage at -2° C or below, was notably poorer than after storage at room temperature in media containing subtoxic concentrations of the cryoprotectants.

Experiment 1.3: Propanediol as a cryoprotectant.

Replacement of glycerol by the same percentage (v/v) of propanediol resulted in a significant increase in the proportion of supercooled embryos developing to expanded blastocysts *in vitro*. Thus, the proportions of embryos which grew into expanded blastocysts dropped from 43% to 17% after 24 h supercooled storage at -5° and -10°, respectively, in media containing glycerol (Table 4), compared to 58% and 52%, respectively, when the supercooled medium contained propanediol (row 1 of Tables 7 and 8). The difference was significant ($P < 0.01$) only at -10°.

Series II

Experiment 2.1: Effects of partial dehydration in the presence of permeating cryoprotectants. The volume changes of mouse embryos exposed to propanediol-sucrose/trehalose solution followed a pattern consisting of initial shrinkage and subsequent gradual expansion. Reexpansion, which is due to the permeation of propanediol and methanol into the embryo, was completed by 6 minutes after placement of the embryos in the hypertonic medium. Therefore, an equilibration period of 8 minutes was adopted in experiments involving supercooling of dehydrated embryos.

The proportion of EB developing into expanded blastocysts *in vitro* after exposure to BM.MP33 or BM.MP66 for 1 h at room temperature was reduced significantly when the concentration of sucrose (Table 5) or trehalose (Table 6) in the cryoprotectant solution exceeded 0.6M ($P < 0.01$). There were no differences due to the particular sugar used; therefore, in subsequent experiments sucrose was used for dehydration.

Experiment 2.2: Supercooling dehydrated embryos.

The concentration of sucrose (0–0.5 M), but not storage temperature, had a significant effect on the survival of early mouse blastocysts in supercooled storage. The addition of sucrose to the supercooling medium increased the ability of EB to withstand supercooled storage: survival after 72 hours' storage increased as the sucrose concentration (i.e. degree of dehydration before cooling) increased from 0.0 to 0.3M ($P < 0.05$), although the effect was not as pronounced when storage was at -

5° (Tables 7 and 8). There was no additional effect when the sucrose concentration was increased beyond 0.3M.

Series III

Development of supercooled whole and demi-embryos *in vivo*

The developmental potential of embryos supercooled for 48 h in BM.MP33 containing 0.3M sucrose was assessed after 24 h in culture and after transfer to pseudopregnant recipients and compared with the development of embryos transferred without supercooling. There was no significant effect of supercooling on the ability of whole embryos to develop into fetuses – development was 35.2% and 40.6%, with and without supercooling, respectively (Table 9). Similarly the *in vivo* development of demi-embryos was not affected by supercooling; 18.4% and 16.7% of supercooled and uncooled demi-embryos, respectively, developed into fetuses after transfer. However, although there was no difference between supercooled intact and demi-embryos in their survival *in vitro*, the demi-embryos, whether supercooled or not, were less successful in establishing pregnancies, on transfer into pseudopregnant hosts, than intact embryos ($P < 0.01$, Table 10).

Table 1. Effect of exposure to methanol on survival of mouse embryos.

Methanol conc[a], % (v/v)	Initial embryo stage CM	EB
6	23/25 (92.0)[a]	26/28 (92.9)[a]
12	22/24 (91.7)[a]	23/25 (92.0)[a]
24	0/25 (00.0)[b]	0/27 (00.0)[b]
48	0/21 (00.0)[b]	N.T.

Values shown are proportions (%) of embryos growing into expanded blastocysts after 1 h exposure to methanol at 25° C.
N.T.= not tested.
Values with different superscripts within columns differ ($P < 0.01$).

Table 2. Effect of exposure to glycerol on survival of mouse embryos

Glycerol conc[a], % (v/v)	Initial embryo stage CM	EB
3	24/25 (96.0)[a]	28/28 (100)[a]
6	25/25 (100)[a]	25/26 (96.2)[a]
12	22/23 (96.7)[a]	23/23 (100)[a]
24	26/28 (92.9)[a]	26/27 (96.3)[a]
48	0/20 (00.0)[b]	N.T.

Values shown are proportions (%) of embryos growing into expanded blastocysts after 1 h exposure to glycerol.
N.T.= not tested.
Values with different superscripts within columns differ ($P < 0.01$).

Table 3. Survival of compacted morula after supercooled storage

	No. of embryos developed/no. of embryos treated (%)											
Temp:	-2° C			-5° C			-10° C			-15° C		
Time:	2	6	24	2	6	24	2	6	24	2	6	24
BM.MG11*	18/30 (60)	12/37 (32)[b]	1/34 (3)	10/29 (35)[b]	7/36 (19)[b]	0/20 (0)	+	+	+	+	+	+
BM.MG33	19/29 (66)	18/42[f] (43)[ab]	2/20 (10)	18/31 (58)[ab]	7/30 (23)[b]	0/22 (0)	3/28 (11)	1/30 (3)	0/27 (0)	+	+	+
BM.MG66	16/26 (62)	22/36[f] (61)[a]	0/21 (0)	22/29 (76)[a]	17/30 (57)[a]	0/20 (0)	10/32 (31)	0/40 (0)	0/25 (0)	5/27 (19)	2/26 (8)	0/28 (0)

Survival expressed as morulae (%) growing into expanded blastocysts after supercooled storage.
*Medium BM.MG66, BM.MG33 and BM.MG11 contained 6%, 3% or 1%, each of methanol and glycerol.
+indicates medium froze during storage.
Values with different superscripts within columns, differ ($P < 0.05$).
[f]Several CM developed into EB during supercooled storage.

Table 4. Survival of early blastocysts after supercooled storage

	No. of embryos developed/no. of embryos treated (%)											
Temp:	- 2 °C			- 5 °C			- 10 °C			- 15 °C		
Time:	2	6	24	2	6	24	2	6	24	2	6	24
BM.MG11*	20/20 (100)	21/21 (100)	15/33 (46)	22/22 (100)	21/28 (75)	5/32 (16)[b]	+	+	+	+	+	+
BM.MG33	20/20 (100)	19/20 (96)	17/33 (52)	21/21 (100)	21/31 (68)	13/32 (41)[a]	26/26 (100)	16/30 (53)	2/21 (10)	+	+	+
BM.MG66	19/20 (95)	16/20 (80)	14/28 (50)	20/21 (95)	23/29 (79)	13/30 (43)[a]	23/24 (96)	16/24 (67)	4/24 (17)	15/22 (68)	7/22 (32)	2/22 (9)

Survival expressed as proportion (%) of early blastocysts growing into expanded blastocysts after supercooled storage.
*Numbers in medium designation indicate the concentrations of methanol and glycerol, e.g 66 = 6% of each, etc.
+indicates medium froze during storage.
Values with different superscripts, within columns, differ ($P < 0.05$).

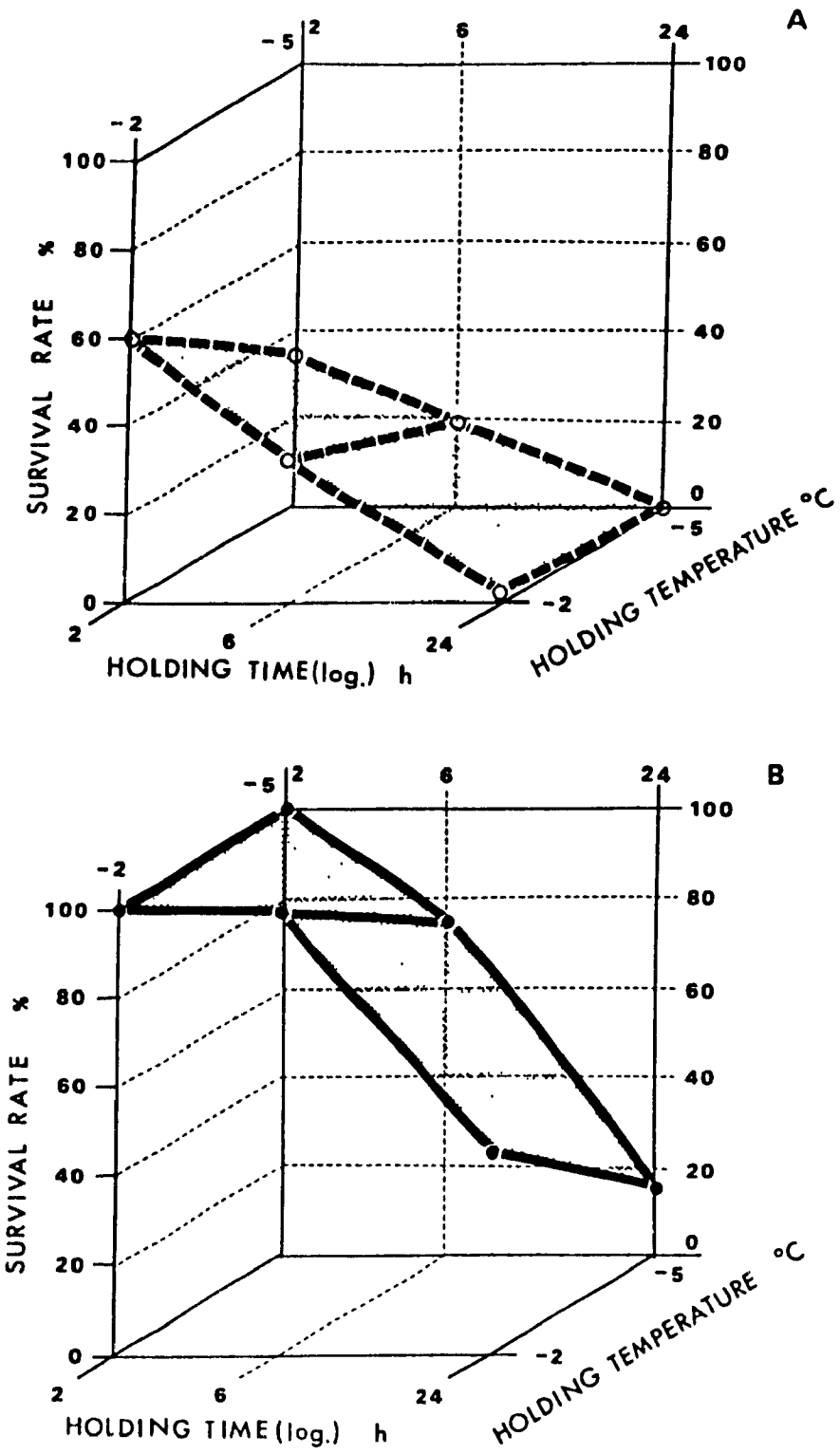


Figure 6. Survival of supercooled compacted morulae (A) and early blastocysts (B)

Embryos were stored in BM.MG11 (basic medium plus 1% each, methanol and glycerol) at various temperatures (z axis) and survival assessed (y axis) by sampling after various storage times (x axis).

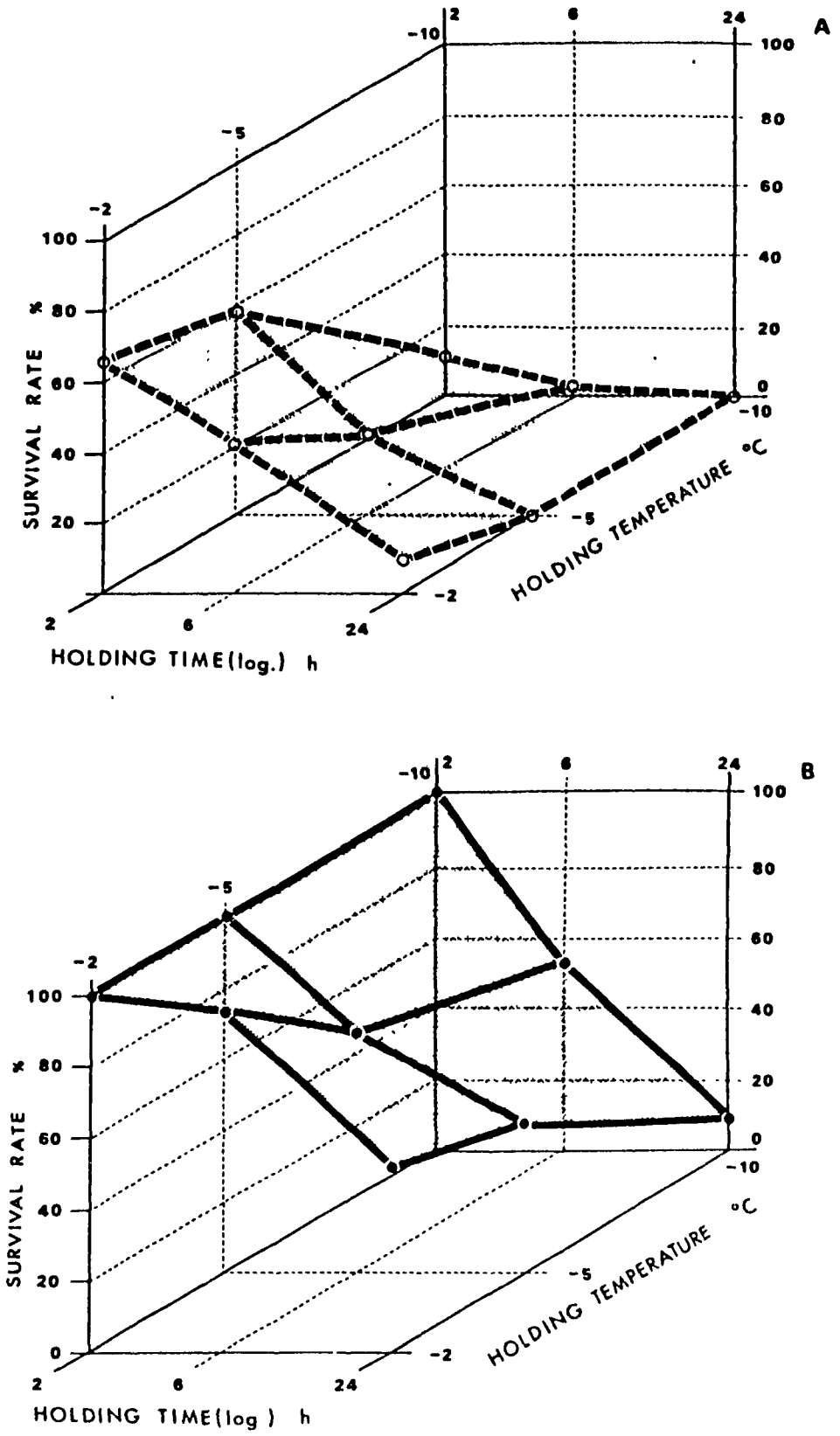


Figure 7. Survival of supercooled compacted morulae (A) and early blastocysts (B)

Embryos were stored in BM.MG33 (basic medium plus 3% each, methanol and glycerol) at various temperatures (z axis) and survival assessed (y axis) by sampling after various storage times (x axis).

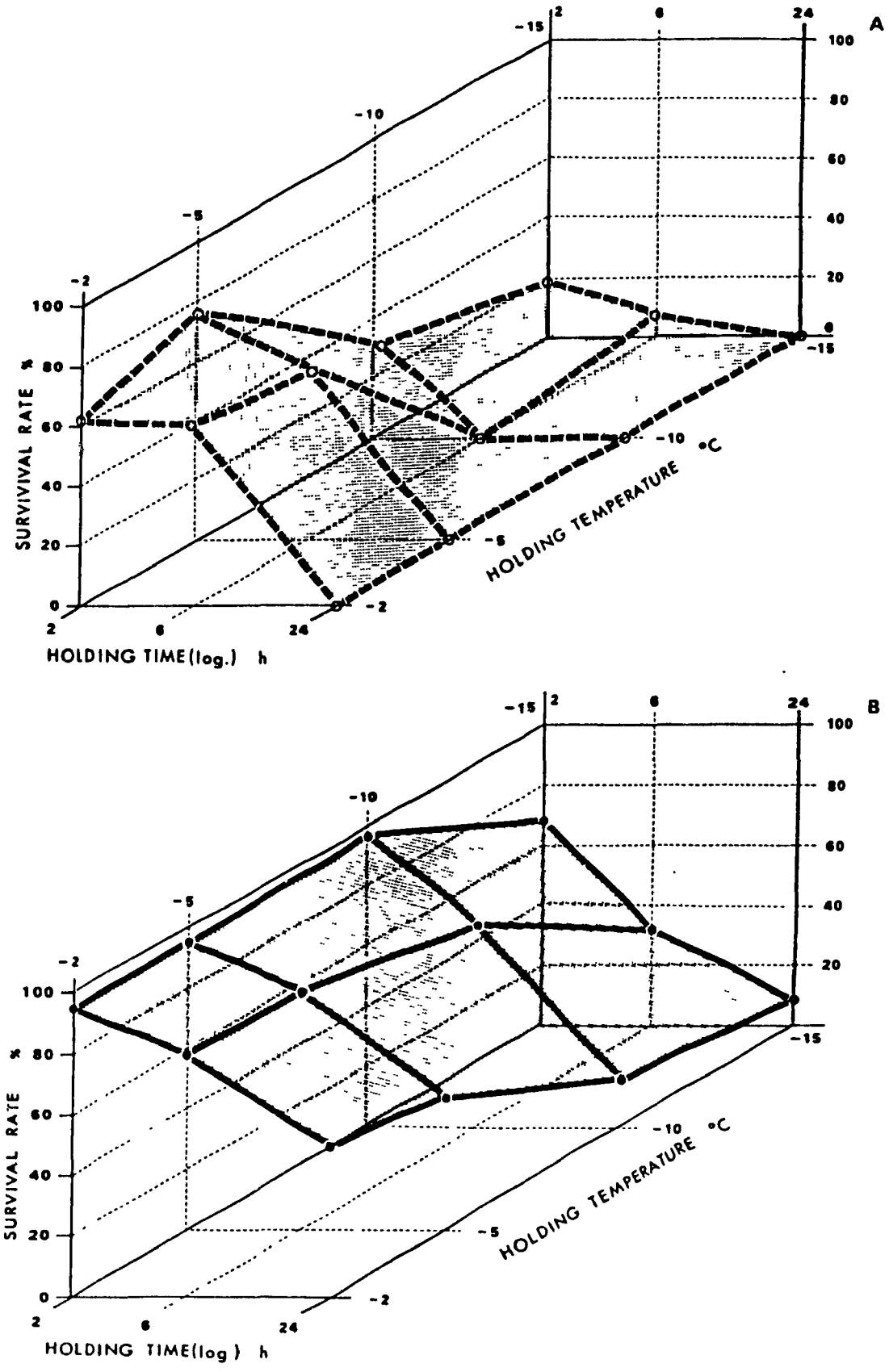


Figure 8. Survival of supercooled compacted morulae (A) and early blastocysts (B)

Embryos were stored in BM.MG66 (basic medium plus 6% each, methanol and glycerol) at various temperatures (z axis) and survival assessed (y axis) by sampling after various storage times (x axis).

Table 5. Effect of exposure to sucrose in the presence of cryoprotectants on the growth of early blastocysts

Sucrose Conc.	Cryoprotectant Medium	
	BM.MP33.	BM.MP66.
0.0 M	20/22 (91)[a]	24/25 (96)[a]
0.1 M	20/21 (95)[a]	23/24 (96)[a]
0.2 M	21/22 (95)[a]	24/25 (96)[a]
0.3 M	21/23 (91)[a]	21/23 (91)[a]
0.4 M	20/22 (91)[a]	20/24 (83)[ab]
0.5 M	19/21 (90)[a]	23/24 (96)[a]
0.6 M	19/22 (86)[a]	26/26 (100)[a]
0.8 M	13/22 (60)[b]	19/26 (73)[b]
1.0 M	7/22 (32)[c]	6/23 (26)[c]

Values shown are proportions (%) of embryos growing into expanded blastocysts after 48 h culture *in vitro*.
Values with different superscripts within columns are different ($P < 0.05$).

Table 6. Effect of exposure to trehalose in the presence of cryoprotectants on the growth of early blastocysts

Trehalose Conc.	Cryoprotectant Medium	
	BM.MP33.	BM.MP66.
0.0 M	27/29 (93)[a]	20/21 (95)[a]
0.1 M	24/26 (92)[a]	22/24 (92)[a]
0.2 M	25/27 (93)[a]	22/22 (100)[a]
0.3 M	30/32 (94)[a]	21/25 (84)[a]
0.4 M	22/23 (96)[a]	20/21 (95)[a]
0.5 M	27/30 (90)[a]	20/21 (95)[a]
0.6 M	21/22 (95)[a]	20/21 (95)[a]
0.8 M	17/25 (68)[b]	11/25 (44)[b]
1.0 M	9/23 (39)[c]	7/22 (32)[c]

Values shown are proportions (%) of embryos growing into expanded blastocysts after 48 h culture *in vitro*. Values with different superscripts within columns differ ($P < 0.05$).

Table 7. Effect of supercooled storage at -5° C on the survival of early blastocysts *in vitro*

(Basic solution BM.MP33 = 3% methanol and 3% glycerol)

Sucrose Conc[a]	24 h.	48 h.	72 h.
0.0 M	15/26 (58)[a]	3/23 (13)[a]	1/24 (4)[a]
0.1 M	16/27 (59)[a]	19/29 (66)[b]	8/20 (40)[b]
0.2 M	17/24 (71)[ab]	16/22 (73)[bd]	12/26 (46)[b]
0.3 M	21/25 (84)[b]	22/24 (92)[d]	15/26 (58)[b]
0.4 M	22/26 (85)[b]	23/25 (92)[d]	15/26 (58)[b]
0.5 M	21/25 (84)[b]	19/22 (86)[bd]	8/21 (38)[b]

Values shown are proportions (%) of embryos growing into expanded blastocysts after 48 h *in vitro*.
Values with different superscripts within columns differ ($P < 0.05$).

Table 8. Effect of supercooled storage at -10° C on the survival of early blastocysts *in vitro*

(Basic solution BM.MP66 = 6% methanol and 6% glycerol)

Sucrose Conc[a]	24 h.	48 h.	72 h.
0.0 M	11/21 (52)[a]	1/22 (5)[a]	0/22 (0)[a]
0.1 M	19/22 (86)[b]	13/21 (62)[b]	6/28 (21)[b]
0.2 M	19/22 (86)[b]	15/21 (71)[b]	11/26 (42)[bd]
0.3 M	24/24 (100)[b]	18/23 (78)[b]	16/29 (55)[d]
0.4 M	22/23 (96)[b]	18/21 (86)[b]	14/30 (47)[d]
0.5 M	20/22 (91)[b]	15/20 (75)[b]	17/31 (55)[d]

Values shown are proportions (%) of embryos growing into expanded blastocysts after 48 h culture.
Values within columns with different superscripts are different ($P < 0.05$).

Table 9. Developmental potential of supercooled embryos *in vitro* and *in vivo*

Embryos	No. of embryos cultured (A)	No. of expanded blastocysts after culture (B) (%)=(B)/(A)	No. of embryos transferred* (C)	No. of live fetuses (D) (%)=(D)/(C)
Whole				
a) not supercooled	-	-	64 (8)	26 (40.6)[a]
b) supercooled	133	102 (76.7)	91 (6)	32 (35.2)[a]
Demi-embryos				
a) not supercooled	-	-	76 (6)	14 (18.4)[b]
b) supercooled	104	78 (75.0)	72 (6)	12 (16.7)[b]

Normal and demi-embryos, produced from embryos at the 2-cell stage and grown in culture to the blastocyst stage, were transferred into pseudopregnant recipients directly or stored at -5° for 48 h, then cultured overnight and transferred.
*Figures in brackets indicate numbers of pseudopregnant recipients.
[a,b]Different superscripts indicate a significant difference ($P < 0.05$).

Table 10. Overall comparisons: Effect of supercooling and embryo integrity on survival and development.

	Development into fetuses (%)	
Supercooled	Demi-embryos	Whole embryos
+	12/72	32/91
-	14/76	26/64
Totals	28/148 (18.9)[b]	58/155 (37.4)[a]

Data shown are taken from Table 9.
[a,b] Figures within row with different superscripts differ ($P < 0.01$).

DISCUSSION

The supercooled solution normally has only a transient existence, created when the medium is cooled and its temperature falls below its freezing point before crystallization can occur. This state is highly unstable and sudden, spontaneous freezing may occur at any instant. This sudden, spontaneous crystallization in greatly supercooled solutions may be damaging to cells because there is no opportunity for dehydration of the cells and intracellular ice formation occurs. The rationale, in designing protocols for supercooled preservation of cells, is to overcome the instability of supercooling media. Zavos and Graham (1981, 1982) adopted emulsification with silicone oil as a means of stabilizing a medium for supercooling turkey sperm suspensions, while Mathias et al. (1984 and 1985) applied a similar approach to stabilizing media for preservation of yeast cells and red blood cells.

Despite success with a variety of cells, this new approach had not been applied to embryos except for a few trials before the present study was initiated (Kasai, 1986, Kasai et al., 1983 and Sun et al., 1989). Embryos present particular problems in cryopreservation because of the relatively large volume of embryos or their component cells, compared to cells which are normally the target of cryopreservation procedures. Because of this difference, the problems of stresses due to osmotic changes, concentrating of electrolytes, ice crystal formation, toxicity of relatively high levels of cryoprotectants, etc., which affect most cells during conventional freezing and thawing procedures, are exacerbated. Because supercooling avoids these problems, it would appear to offer suitable means of preserving embryos. Our findings demonstrate that preservation of embryos at low temperature, without freezing, that is, storage in a supercooled state, offers a means of preserving mouse embryos, albeit, so far, for a limited period.

In considering suitable cryoprotectants, glycerol and methanol were chosen because these had already been shown to be effective in protecting mouse embryos

during freezing (Whittingham et al., 1972; Rall et al., 1984). Although these permeating solvents were claimed to have low toxicity, no data were provided. Consequently, the toxicity of these reagents was tested, using concentrations considered to be potentially useful for supercooling. CM and EB tolerated exposure for 1 h to up to 12% methanol or 24% glycerol at room temperature (see Tables 1 and 2). Our observations are in agreement with the findings of Kasai et al. (1990) who reported that 40% glycerol was toxic to mouse embryos exposed to it for 20 min at 20° C, and of Rall et al. (1984) who reported that exposure of day-4 mouse embryos to 3 M methanol for 3 h at room temperature was not deleterious to subsequent development *in vitro*, while 4 M methanol had a significant negative effect. However these observations offer no information as to the tolerance of the embryos to the cryoprotectants for longer periods, even at lower concentrations and/or lower temperatures and do not permit distinguishing possible intolerance to long exposure to the cryoprotectants from other damaging influences (see below).

We found a significant difference between CM and EB in their ability to withstand supercooled storage at -2 to -15° C. Early blastocysts showed significantly higher survival than morulae ($P < 0.01$). Moreover, the significant interaction ($P < 0.05$) between embryo stage and the effect of preservation temperature on survival implies that the sensitivity of embryos to various subzero temperatures changes with the developmental state. These results agree with previous reports that tolerance to cooling increases with the development of the embryo. This has been attributed to a gradual increase in the permeability of the cell, which allows increasing penetration of cryoprotectants into the cell as the embryo's developmental stage advances (Mazur, 1970; Mazur et al. 1976). While such an interpretation may be valid in the case of embryos undergoing freezing, it is unclear whether this is relevant in the present study. Here, ice crystallization and associated osmotic changes do not occur, at least at the more moderate subzero temperatures, and the advantage to the cell from having increased intracellular concentrations of cryoprotectants is not immediately apparent. We also have to

consider the osmotic shock that results from removal of the cryoprotectants after treatment because embryo permeability has a profound effect on this osmotic shock. Nevertheless, the blastocyst may be the stage of mouse embryo most amenable to supercooled storage.

Methanol and glycerol have long been known to protect against the deleterious effects of freezing and thawing (Lovelock, 1953). The use of propanediol, in combination with glycerol, has been found to be advantageous in both the cryopreservation (-196° C) and hypothermal storage (+4° C) of erythrocytes (Boutron et al., 1984; Vorotilin et al., 1989). Ko and Threlfall (1988) found that freshly ovulated mouse oocytes are well preserved after storage at -196°C in the presence of propanediol. Propanediol has also been reported to substantially increase the survival of hamster oocytes subjected to rapid freezing and thawing (Hyatt Sachs et al., 1989). Hernandez-Ledezma and Wright, Jr (1989) reported that a higher percentage of 1-cell embryos developed to the 2-cell stage after freezing and thawing in the presence of propanediol than in glycerol, dimethylsulfoxide, or in a combination of 1 M propanediol and 0.5 M glycerol. In the present study also, the replacement of glycerol with propanediol proved beneficial (discussed below).

The finding that several CM formed a small blastocoel during storage at -2° C (see Table 3, footnote) clearly shows that metabolism is not arrested at this temperature. However, it is necessary to make a further observation on this blastocoel formation in order to determine the developmental potential *in vivo*. Although it was expected that lower temperatures would suppress metabolism more effectively, lower temperatures did not increase the survival time of embryos stored in our original media formulations (cf. bottom row in Tables 3 and 4). One important cause to be considered is the disruption of the organization of macromolecules, particularly proteins, at low temperatures. Ova and embryos normally contain approximately 18 % (w/v) dry matter, of which 65 to 70 % is protein (Brinster, 1963). Accordingly, changes which seriously affect the structure of proteins would be expected to have profound effects on the viability of eggs and embryos.

Although the phenomenon of heat denaturation of proteins is well known, less is understood about the behaviour of proteins at low temperatures. The main reason for this is that the solvent medium often freezes in the temperature range of interest, obscuring observations. Nevertheless, there are examples of cold denaturation of proteins, whereby a protein spontaneously unfolds or subunits dissociate into inactive forms. Such denaturations may not be completely reversible as proteins may not refold properly or reassemble when physiological conditions are restored (Brandt, 1967; Brandt et al., 1970).

Another important possibility is the occurrence of "cold shock", which is attributed to phase changes which may occur in various membrane lipids during cooling (Watson, 1981), even when there is no water/ice phase change in the system. This phenomenon is particularly conspicuous in porcine spermatozoa which are extraordinarily sensitive to temperature reduction. Moreover, electron and light microscopy studies of early bovine embryos (8–16 cells) showed that in embryos cooled to 4° C, the distribution of organelles within the blastomeres was disrupted whereas the morphology of the organelles appeared unaltered (Mohr and Trounson, 1982). Quinn (1985) also speculated that the damage caused to cells that are sensitive to low temperature may be associated with lipid phase changes within the cell membranes and proposed a lipid phase separation model to explain the damage caused to biological membranes exposed to low temperatures.

These initial findings encouraged the continued exploration of supercooling as an alternative to conventional freezing-thawing preservation methods, and subsequent work was directed toward increasing survival during extended low temperature storage (24 to 72 h). Inasmuch as early blastocysts were maintained in supercooled storage more successfully than compact morulae, further studies were applied to blastocysts only, using methanol and propanediol as the cryoprotectants.

In order to increase survival at lower temperatures, we investigated the effects of dehydrating the embryos by adding non-permeating compounds such as sucrose and

trehalose to the preservation medium to increase its osmolarity. Renard et al. (1984) showed that partial dehydration of 2-cell rabbit embryos by sucrose, allowing equilibration for up to 15 min at room temperature, permitted a higher survival after rapid freezing and thawing. Szell and Shelton (1986) reported that the dehydration of embryos caused by increased tonicity of the medium by addition of sucrose was beneficial in mouse embryos frozen rapidly in liquid nitrogen vapour. Recently, Fiser et al. (1991) demonstrated that dehydrated boar sperm survive supercooled storage substantially better than sperm with all their intracellular water present. In the present study, exposure of EB to media containing more than 0.6 M sucrose or trehalose for 1 h, *without* supercooled storage, significantly decreased the proportion developing to expanded blastocysts in culture ($P < 0.01$). Our results are similar to those of Kasai et al.(1981) and of Szell and Shelton (1986) who found that exposure to high concentrations of sucrose significantly reduced the developmental potential of mouse embryos. In both of these studies, it was found that the deleterious effects of exposure to sucrose were reduced when the exposure temperature was lowered. The toxic effects of exposure to high concentrations of either sucrose or trehalose may be due to excessive dehydration, resulting in distortion of structures beyond their plastic limits (Steponkus and Gordon, 1985), precipitation of vital cytoplasmic constituents or alteration of intracellular pH or combinations of these effects, producing irreversible changes . The reduction of the deleterious effects at lower temperature may simply reflect significantly slower exit of water from the embryo cells.

The pattern of volume changes in mouse embryos observed microscopically during equilibration with hypertonic media (see Results, Experiment 2.1) agreed with the predictions of Leibo (1983, 1984) and Schneider and Mazur (1984), who explained the pattern as follows. In a hypertonic solution of cryoprotectant, the cell initially loses water and shrinks to compensate for the high osmolarity produced by glycerol and sucrose in the extracellular compartment; the relatively rapid shrinkage is followed by a gradual increase in volume as glycerol enters and water re-enters the cell; the equilibration is

complete when no further volume changes occur. In the present study, propanediol and trehalose would behave in a similar manner, replacing glycerol and sucrose, respectively.

Frozen–thawed embryos survive only when a permeating cryoprotectant is added to the suspending medium. Sucrose alone does not afford adequate protection. In conventional embryo cryopreservation, sucrose in the medium causes exosmosis of water, so that the likelihood of intracellular ice formation is reduced and the embryos can be frozen rapidly and without seeding (Renard et al., 1984; Takeda et al., 1984). These authors found the optimal concentration of sucrose in the freezing medium to be 0.25–0.5M. This is similar to the concentrations found to be most useful in the present study on supercooling. This suggests that dehydration beyond the degree caused by these concentrations of sugar is not beneficial or, at best, offers no additional benefit to the cell.

A few attempts have been made to preserve mouse embryos at temperatures above freezing (0°) by using protective agents. Kasai et al. (1981, 1983) reported that sucrose had a protective effect on mouse embryos stored at 0° C for 24–120 h. Moreover, survival was greater when the embryos were stored in 0.25 to 0.75M sucrose than in 0.1 or 1 M sucrose. While the negative effect of the highest concentration of sucrose may be accounted for by the explanation offered above, the basis for the beneficial action of intermediate concentrations is unknown.

In the third series of experiments, we found that preservation of embryos in the supercooled state did not affect the subsequent growth *in vitro* or *in vivo*, whether of whole or demi–embryos. These findings differ from those of Heyman (1985) who found survival of transferred frozen/thawed bovine demi–embryos was significantly lower than that of fresh demi–embryos (20% and 36.3%, respectively). However, our observations are similar to those of Kobayashi et al. (1990) who found no effect of vitrification (which represents an extreme case of supercooling) on post–thaw development *in vitro* of rabbit whole or half– morulae, nor did they find a difference between the proportions of whole and bisected morulae growing into blastocysts after vitrification. Although these workers

suggested that the vitrification method of cryopreservation is more successful with bisected rabbit embryos than conventional freezing methods, they assessed the survival of demi-embryos *in vitro* only. Because of this difference as well as the species differences precise comparison of our findings with those of Kobayashi et al. is not possible. It is important to note another difference. In the present study, cryopreserved embryos were transferred into pseudopregnant hosts only after culture for 24 h, whereas Kobayashi transferred whole, cryopreserved, rabbit morulae after only 3 h in culture. Immediate transfer after thawing has been the universal practice because the viability of cryopreserved embryos is believed to deteriorate during prolonged (i.e. longer than a few hours) post-thaw culture. This was not the case with supercooled whole or demi-embryos (cf Table 9), indicating that supercooled cryopreservation did not damage embryos in ways which are readily detected solely by culture *in vitro* or by transfer. However, culturing embryos after they have been treated for >> 3 hours permits selection of only those embryos that have survived and developed for transfer.

Inspection of the original raw data concerning the rate of pregnancy following transfer of whole or demi-embryos, with or without supercooling, suggests a certain correlation between the number of demi-embryos transferred and the resultant pregnancy rate. When 9 demi-embryos were transferred into each pseudopregnant recipient, none became pregnant (i.e. no fetuses were found). However, when 12 or more demi-embryos were transferred into each recipient, an average of 19% developed into fetuses (see Appendix B, Tables B-3, B-4) except for one recipient transferred 12 demi-embryos (#5 Table B-4). Consequently, 12 demi-embryos seems to be the threshold number required to stimulate corpus luteum function and maintain pregnancy to term. It is noteworthy that the rate of development of transferred whole embryos was twice that of the demi-embryos. This relation would be expected if the threshold is determined by the mass of embryonic tissue rather than the number of embryonic structures participating in luteal "rescue". Thus, Tarkowski (1959 and 1967) and Rands (1985) showed that after manipulation of the size of embryos by destroying blastomeres or by aggregating

embryos, reduction of cell number lead to premature luteolysis. In the same vein, Hogan et al. (1986) recommended that 8 to 16 embryos should be transferred for assessment of survival *in vivo*, but, in view of the foregoing deliberations, these numbers would not apply in the case of demi-embryos. The foregoing strongly suggests that the threshold effect is of particular significance in the case of bovines where transferred frozen demi-embryos show poor survival and increasing the number of demi-embryos per recipient is not feasible (except when the two halves of one embryo only are transferred, avoiding the possibility of freemartin production from conjoined development of fetuses of different sex). One possible remedy to the problem is co-transfer of additional embryonic material such as trophoblastic vesicles (Heyman, 1985).

The supercooled storage technique, developed for mouse embryos, may also prove to have similar benefits for the cryopreservation of the embryos of domestic animals. However the protocols worked out in our research represent only a relatively early stage in the development of a new cryopreservation technology.

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APPENDIX A: Media Formulations

Table A-1. Basic supercooling medium (BM).

	g/100 ml
BSA	0.28
Dextran	1.58
Glucose	1.32
Glycine	0.12
Proline	0.25
Ribose	0.75
Sorbitol	0.28
TES (M)[a]	8.8 ml of M sol[a]
Trehalose	1.26
TRIS (THAM)	0.35
H_2O	to 100 ml

Adjust to pH 7.0 with M TES before making up to 100 ml.
Osmolarity = 330 ± 10 mOsm.

Table A-2. Preparation of M16 (culture medium)
(Whittingham, 1971; gentamycin used instead of original penicillin + streptomycin)

Compound	mM	Molecular weight	g/liter
NaCl	94.66	58.450	5.533[a]
KCl	4.78	74.557	0.356
$CaCl^2:2H^2O$	1.71	147.200	0.252
KH_2PO_4	1.19	136.091	0.162
$MgSO_4:7H_2O$	1.19	246.500	0.293
$NaHCO_3$	25.00	84.020	2.101
Sodium lactate	23.28	112.100	2.610 (= 4.349 ml of 60% syrup)
Sodium pyruvate	0.33	110.000	0.036
Glucose	5.56	179.860	1.000
Bovine serum albumin (BSA)			4.000
Gentamycin sulfate*			25 μg/ml
Phenol Red			0.010
H_2O			to 1 liter

[a]Increase NaCl to 5.68 when $CaCl_2$ omitted for Ca** free medium.
*as a replacement for penicillin/streptomycin specified by Whitingham

Table A-3. Preparation of M2 (manipulation medium) (Fulton and Whittingham, 1978; penicillin + streptomycin replaced by gentamycin)

Compound	mM	Molecular weight	g/liter
NaCl	94.66	58.450	5.533[a]
KCl	4.78	74.557	0.356
$CaCl_{2.2H}2O$	1.71	147.200	0.252
KH_2PO_4	1.19	136.091	0.162
$MgSO_4:7H_2O$	1.19	246.500	0.293
$NaHCO_3$	4.15	84.020	0.349
HEPES	20.85	238.300	4.969
Sodium lactate	23.28	112.100	2.610 (4.349 ml of 60% syrup)
Sodium pyruvate	0.33	110.000	0.036
Glucose	5.56	179.860	1.000
Bovine serum albumin (BSA)			4.000
Gentamycin			25 μg/ml
Phenol Red			0.010
2x glass-distilled H_2O			to 1 liter

[a] NaCl is increased to 5.68 when $CaCl_2$ and $MgSO_4$ are omitted.

Table A-4. Preparation of M2 and M16 from concentrated stock solutions

Fresh media were prepared every week from concentrated stock solutions, as indicated below:

	Component	g/100ml	M2 (50ml)	M16 (10ml)
Stock A (10x)	NaCl	5.534		
	KCl	0.360		
	KH_2PO4	0.162		
	$MgSO_4:7H_2O$	0.294	5.0	1.0
	Sodium lactate	4.439 ml of 60% syrup.		
	Glucose	1.000		
	Gentamycin	0.025		
Stock B (10x)	$NaHCO_3$	0.2106 g/10 ml	0.8	1.0
Stock C (10x)	Na pyruvate	0.036 g/10 ml	0.5	0.1
Stock D (10x)	$CaCl^2:2H^2O$	0.252 g/10 ml	0.5	0.1
Stock E (10x)	HEPES (M sol^n of Na salt)		20.85 ml/100ml	4.2
Add before adjusting to final volume:	Phenol red		0.5 mg	0.1 mg
	Bovine serum albumin(BSA)		200 mg	40 mg
	H_2O to final volume		(39 ml)	(7.8 ml)

Sterilize stock solutions by filtration using 0.22 μm pore membrane filter. Stocks A,D,and E can be kept at 4° C up to 3 months. Stock C should be replaced every 2 weeks. Filter the complete medium and store at 4° C for no more than 1 week.

(Hogan et al., 1986)

APPENDIX B: Raw Data

Table B-1. Development of whole embryos *in vivo*; no supercooling

Recipient	No. of embryos transferred (A)	Pregnant	No. of implantation sites	No. of live fetuses (%) (B) (%) = (B)/(A)
# 1	8	+	1	4 (50.0)
# 2	8	-	2	0 (00.0)
# 3	8	+	2	4 (50.0)
# 4	8	+	1	3 (37.5)
# 5	8	-	0	0 (00.0)
# 6	8	+	0	5 (62.5)
# 7	8	+	1	5 (62.5)
# 8	8	+	2	5 (62.5)
Total	64	6/8	9	26 (40.6)
	48 in pregnant recipients			26 (54%)

Table B-2. Development *in vivo* of supercooled whole embryos

Recipients	No. of embryos transferred (A)	Pregnant	No. of implantation sites	No. of live fetuses (%) (B) (%) = (B)/(A)
# 1	16	+	6	7 (43.8)
# 2	16	+	4	8 (50.0)
# 3	16	+	5	6 (37.5)
# 4	16	-	6	0
# 5	14	+	5	7 (50.0)
# 6	13	+	7	6 (46.2)
Total	91	5/6	33	34 (37.4)
	75 in pregnant recipients			34 (45%)

Table B-3. Development *in vivo* of demi-embryos: not supercooled

Recipient	No. of embryos transferred (A)	Pregnant	No. of implantation sites	No. of live fetuses (%) (B) (%) = (B)/(A)
# 1	12	-	2	0 (00.0)
# 2	12	-	1	0 (00.0)
# 3	12	+	0	5 (41.7)
# 4	12	+	1	3 (25.0)
# 5	13	+	2	4 (30.8)
# 6	15	+	5	2 (13.3)
Total	76	4/6	11	14 (18.4)
	52 in pregnant recipients			14 (27%)

Table B-4. Development of supercooled demi-embryos *in vivo*

Recipient	No. of embryos transferred (A)	Pregnant	No. of implantation sites	No. of live fetuses (%) (B) (%) = (B)/(A)
# 1	9	-	1	0 (00.0)
# 2	13	+	4	4 (30.8)
# 3	9	-	0	0 (00.0)
# 4	13	+	0	6 (46.2)
# 5	12	-	0	0 (00.0)
# 6	16	+	4	2 (12.5)
Total	72	3/6	9	12 (16.7)
	42 in pregnant recipients			12 (29%)

Appendix C: Statistical Analyses

SAS General Linear Models; program for Digital VAX computer.

```
FILE NAME IN '[AG150GET. 1KU]FUKU. DAT';
DATA F1;
INFILE IN;
INPUT EXP 1-2 MOR 4-5 REP 7 BSOL $ 9-10 TIME 12-13 SURV 18-19 TOT 21-2
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DATA; SET F1;
*;
TITLE 'EXPERIMENT NUMBER 1 AND 2 (SUCROSE VS TREHALOSE)';
*;
IF EXP GT 2 THEN DELETE;
*;
PSURV=SURV/TOT;
*;
PROC GLM;
CLASS EXP MOR BSOL;
MODEL PSURV=EXP MOR BSOL EXP*MOR EXP*BSOL MOR*BSOL/SS3;
*;
PROC GLM;
CLASS EXP MOR BSOL;
MODEL PSURV=EXP MOR BSOL/SS3;
ISMEANS EXP MOR BSOL/PDIFF STDERR;
*;
DATA; SET F1;
*;
TITLE 'EXPERIMENT NUMBER 3 AND 4 (SUPERCOOLING -5 AND -10 C)';
*;
IF EXP LT 3 OR EXP GT 4 THEN DELETE;
*;
PSURV=SURV/TOT;
*;
PROC GLM;
CLASS EXP MOR TIME;
MODEL PSURV=EXP MOR TIME EXP*MOR EXP*TIME MOR*TIME/SS3;
*;
PROC GLM;
CLASS EXP MOR TIME;
MODEL PSURV=EXP MOR TIME MOR*TIME/SS3;
ISMEANS EXP MOR TIME MOR*TIME/PDIFF STDERR;
*;
DATA F1;
INFILE IN;
INPUT EXP 1-2 STAGE 4-5 REP 7 BSOL $ 9-10 CONC 12-13 SURV 18-19 TOT 21-2
*;
TITLE 'EXPERIMENT NUMBER 5 AND 6 (METHANOL AND GLYCEROL TOXICITY)';
*;
IF EXP LT 5 OR EXP GT 6 THEN DELETE;
*;
PSURV=SURV/TOT;
```

```
*;
PROC GLM;
CLASS EXP STAGE CONC;
MODEL PSURV=EXP STAGE CONC EXP*STAGE EXP*CONC STAGE*CONC/SS3;
*;
PROC GLM;
CLASS EXP STAGE CONC;
MODEL PSURV=EXP STAGE CONC/SS3;
ISMEANS EXP STAGE CONC/PDIFF STDERR;
*;
DATA F1;
INFILE IN;
INPUT EXP 1-2 STAGE 4-5 BSOL $ 9-10 TEMP 12-13 TIME 15-16 SURV 18-19 T
21-22;
*;
TITLE 'EXPERIMENT NUMBER 7 (MOUSE EMBRYO)';
*;
IF EXP NE 7 THEN DELETE;
*;
PSURV=SURV/TOT;
*;
PROC GLM;
CLASS STAGE BSOL TEMP TIME;
MODEL  PSURV=STAGE  BSOL  TEMP  TIME  STAGE*BSOL  BSOL*TEMP  BSOL*TI
TEMP*TIME/SS3;
*;
PROC GLM;
CLASS STAGE BSOL TEMP TIME;
MODEL PSURV=STAGE BSOL TEMP TIME STAGE*TEMP STAGE*TIME TEMP*TIME/SS3;
ISMEANS STAGE BSOL TEMP TIME STAGE*TEMP STAGE*TIME TEMP*TIME/PDIFF STDER
*;
DATA F1;
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PUT EXP 1-2 TYPE 4-5 PROCESS 7 BSOL & 9-10 CONC 12-13 SURV 18-19 TOT 2
22;
*;
TITLE 'EXPERIMENT NUMBER 8 (in vivo VIABILITY)';
*;
IF EXP NE 8 THEN DELETE;
*;
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*;
PROC GLM;
CLASS TYPE PROCESS;
MODEL PSURV=TYPE PROCESS/SS3;
ISMEANS TYPE PROCESS/PDIFF STDERR;
```

SAS χ^2 analysis; program for VAX computer

```
FILE NAME IN '[AG150GET. 1KU]FUKU. DAT1';
DATA F1;
INFILE IN;
INPUT EXP 1-2 MOR 4-5 REP 7 BSOL $ 9-10 TIME 12-13 PSURV 18;
*;
DATA; SET F1;
*;
TITLE 'EXPERIMENT NUMBER 1 AND 2 (SUCROSE VS TREHALOSE)';
*;
IF EXP GT 2 THEN DELETE;
*;
PROC FREQ;
TABLES PSURV*EXP/CHISQ;
PROC FREQ;
TABLES PSURV*MOR/CHISQ;
PROC FREQ;
TABLES PSURV*BSOL/CHISQ;
PROC FREQ;
TABLES PSURV*EXP*MOR/CHISQ;
PROC FREQ;
TABLES PSURV*MOR*BSOL/CHISQ;
*;
DATA; SET F1;
*;
TITLE 'EXPERIMENT NUMBER 3 AND 4 (SUPERCOOLING -5 AND -10 C)';
*;
IF EXP LT 3 OR EXP GT 4 THEN DELETE;
*;
PROC FREQ;
TABLES PSURV=EXP/CHISQ;
PROC FREQ;
TABLES PSURV*MOR/CHISQ;
PROC FREQ;
TABLES PSURV*TIME/CHISQ;
PROC FREQ;
TABLES PSURV*EXP*MOR/CHISQ;
PROC FREQ;
TABLES PSURV*MOR*TIME/CHISQ;
*;
DATA F1;
INFILE IN;
INPUT EXP 1-2 STAGE 4-5 REP 7 BSOL $ 9-10 CONC 12-13 PSURV 18;
*;
TITLE 'EXPERIMENT NUMBER 5 AND 6 (METHANOL AND GLYCEROL TOXICITY)';
*;
IF EXP LT 5 OR EXP GT 6 THEN DELETE;
*;
PROC FREQ;
TABLES PSURV*EXP/CHISQ;
PROC FREQ;
TABLES PSURV*STAGE/CHISQ;
PROC FREQ;
TABLES PSURV*CONC/CHISQ;
*;
DATA F1;
```

```
INFILE IN;
INPUT EXP 1-2 STAGE 4-5 BSOL $ 9-10 TEMP 12-13 TIME 15 16 PSURV 18;
*;
TITLE 'EXPERIMENT NUMBER 7 (MOUSE EMBRYO)';
*;
IF EXP NE 7 THEN DELETE;
*;
PROC FREQ;
TABLES PSURV*STAGE/CHISQ;
PROC FREQ;
TABLES PSURV*BSOL/CHISQ;
PROC FREQ;
TABLES PSURV*TEMP/CHISQ;
PROC FREQ;
TABLES PSURV*TIME/CHISQ;
PROC FREQ;
TABLES PSURV*STAGE*TEMP/CHISQ;
PROC FREQ;
TABLES PSURV*STAGE*TIME/CHISQ;
PROC FREQ;
TABLES PSURV*TEMP*TIME/CHISQ;
*;
DATA F1;
INFILE IN;
INPUT EXP 1-2 TYPE 4-5 PROCESS 7 BSOL & 9-10 CONC 12-13 PSURV 18;
*;
TITLE 'EXPERIMENT NUMBER 8 (in vivo VIABILITY)';
*;
IF EXP NE 8 THEN DELETE;
*;
PROC FREQ;
TABLES PSURV*TYPE/CHISQ;
PROC FREQ;
TABLES PSURV=PROCESS/CHISQ;
```

Coding of Data for SAS ANALYSIS:

Prototypic Example: Tolerance to sucrose (Experiment 2.1)

Column 1: Trial
2: Sucrose concentration (M/10)
3: Replicate
4: Supercooling medium
5: No. of survivors
6: No. of embryos

1	0	1	33	10	12
1	0	2	33	10	10
1	1	1	33	10	10
1	1	2	33	10	11
1	2	1	33	12	12
1	2	2	33	9	10
1	3	1	33	11	12
1	3	2	33	10	11
1	4	1	33	10	12
1	4	2	33	10	10
1	5	1	33	8	9
1	5	2	33	11	12
1	6	1	33	11	12
1	6	2	33	8	10
1	8	1	33	8	12
1	8	2	33	5	10
1	10	1	33	4	12
1	10	2	33	3	10
1	0	1	66	12	13
1	0	2	66	12	12
1	1	1	66	12	13
1	1	2	66	11	11
1	2	1	66	13	13
1	2	2	66	11	12
1	3	1	66	8	9
1	3	2	66	13	14
1	4	1	66	10	12
1	4	2	66	10	12
1	5	1	66	10	10
1	5	2	66	13	14
1	6	1	66	14	14
1	6	2	66	12	12
1	8	1	66	10	14
1	8	2	66	9	12
1	10	1	66	2	8
1	10	2	66	4	15