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XENOGENOUS FERTILIZATION OF CRYOPRESERVED GOLDEN HAMSTER AND SQUIRREL MONKEY OVA

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XENOGENOUS FERTILIZATION OF CRYOPRESERVED GOLDEN HAMSTER AND SQUIRREL MONKEY OVA

By

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ABSTRACT

XENOGENOUS FERTILIZATION OF CRYOPRESERVED GOLDEN HAMSTER AND SQUIRREL MONKEY OVA

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The fertilization of frozen hamster and squirrel monkey ova in the rabbit oviduct was the objective of this investigation. A total of 2,842 (87.0%) of the 3,267 hamster ova were judged viable by their ability to exclude trypan blue after cryopreservation. Analysis of the factors involved in the freezing of hamster ova showed that successful hamster ova cryopreservation can be accomplished using PBS or TC-199 as the freezing medium, 1.5 M or 2.0 M DMSO as the cryoprotectant, initial slow cooling to temperatures of -10°C, -20°C, or -30°C, terminal slow cooling temperatures of -40°C to -80°C, and recovery of the stored ova by thawing at 1-4°C/min or 92°C/min. Cryopreserved hamster ova were fertilized at a significantly lower rate (11%) than the nonfrozen controls (35.7%).

Cryopreservation of squirrel monkey ova by the same procedure as hamster ova showed significantly lower viability. The viability of cryopreserved squirrel monkey ova was increased by adding DMSO at 20°C instead of at 0°C. However, viability of cryopreserved squirrel monkey ova was not affected by increasing the concentration of DMSO from 1.5 M to 2.0 M or 3.0 M added at 20°C. Recovery of frozen

squirrel monkey ova at rates of 1-4°C/min, 17.6-27.6°C/min, or 96°C/min did not alter the viability of these ova. Frozen-thawed squirrel monkey ova yielded xenogenous and *in vitro* fertilization rates similar to those of the nonfrozen controls.

To my grandmother,

Katherine T. Weber

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INTRODUCTION

The production of human offspring by *in vitro* fertilization and embryo transfer has heralded the need for a non-human primate model to study the morphological, physiological, and biochemical events of early development. Such a primate model could be used to screen for pharmacological or environmental factors which might be beneficial or detrimental to the developing embryo. The development of techniques for the nonsurgical recovery of primate ova and the extracorporeal (*in vitro* and xenogenous fertilization) production of primate embryos has aided in the study of primate development and the establishment of this screening system.

The efficacy of *in vitro* fertilization and xenogenous fertilization systems, to study primate development, would be enhanced by a readily accessible pool of ova for use in the developmental studies. The intent of the present investigations was to develop a system for the cryopreservation of squirrel monkey (*Saimiri sciureus*) ova. In order to avoid loss of expensive primate ova, preliminary cryopreservation trials utilized golden hamster ova.

Since the purpose of this investigation was to preserve the viability of ova for eventual fertilization, the viability of the frozen-thawed ova was tested by the ability of the ova to be fertilized by homologous sperm in the oviduct of the pseudopregnant rabbit (xenogenous fertilization).

LITERATURE REVIEW

Cryopreservation

A Historical Perspective:

The first report of the successful storage of a fertilized mammalian embryo at a temperature below the normal body temperature for the species was by Chang (1947). One cell rabbit embryos, which were held at 10°C or 5°C for 120 to 144 hours, cleaved after being in culture at 37°C for 24 hours and yielded live offspring after transfer. The optimum temperature for storage of rabbit embryos held in rabbit serum was reported to be 10°C. Furthermore, these stored embryo were viable after being stored for 144 to 168 hours (Chang, 1948a,b,c)

Early attempts at freezing rabbit ova and embryos to the temperature of -76°C by rapid cooling and thawing met with complete destruction of the ova and embryos (Smith, 1949). However, a small percentage of rabbit embryos (6/556) did continue to develop after slow cooling to -79°C or to -190°C and rapid thawing when the freezing medium was supplemented with 15% glycerol (v/v) (Smith, 1952; 1953). But the development was minimal since only one ova developed to the 6-cell stage (Smith, 1952).

Furthermore, the addition of glycerol to the storage medium of unfertilized mouse ova was shown to aid in their cold storage at 5°C (Lin *et al.*, 1957). Unfertilized mouse ova were stored at -10°C in

5% glycerol in saline for times of 0.25 to 2 hours. Survival was limited and decreased with prolonged storage (Sherman and Lin, 1959). These mouse ova, which were able to survive storage at -10°C, were transplanted to mated females and did result in live births (Sherman and Lin, 1958a,b). Sherman and Lin (1958b) observed in some cases that mouse ova which were frozen to -10°C showed intracellular freezing. Upon thawing, these ova showed severe damage. In 1963, Sherman showed that this intracellular ice formation could be avoided by slower cooling of the ova. When mouse ova were cooled to -20°C at a rate of 0.7°C/min, 90% were structurally unaltered as compared to those frozen at a more rapid rate. Furthermore, when rabbit embryos were slow cooled in 10% glycerol to -79°C, a high proportion of embryos developed to birth after thawing and embryo transfer (Ferdows et al., 1958).

The need for the addition of glycerol to the storage medium and the beneficial effects of slow cooling on oocyte survival after storage at subzero temperatures have also been demonstrated by the viability of grafted ovarian tissue after low temperature storage. Rat ovarian tissue was soaked in 15% glycerol and cooled to -79°C or -190°C. After thawing, the ovarian tissue was grafted to recipient animals and the endocrine activity of the tissue resumed. The resumption of activity only occurred when 15% glycerol was present in the freezing medium and slow cooling was employed (Smith et al., 1951; Parkes et al., 1953; Parkes, 1958). And although the endocrine activity of the ovarian tissue was restored, a high proportion of the oocytes in the ovarian grafts were destroyed (as many as 90%) (Parkes and Smith, 1953; Deanesly, 1954, 1957; Green et al., 1956). When the

same experiments were done in mice, Parrott (1960) observed similar results. However, even though a great deal of oocyte destruction occurred, a small proportion of the graft-recipient mice were fertile. But the extent of reproductive life in these mice was greatly reduced.

The early attempts at preserving mammalian embryos met with limited success until 1971 when Whittingham reported that 55% to 65% of mouse eight cell embryos and early blastocysts suspended in 7.5% pyrolidine in a modified Dulbecco's phosphate buffered saline (PBS) survived freezing to -79°C. This method allowed for storage for only 30 minutes and the results have not been duplicated. Then, in 1972, Whittingham et al. evaluated the factors involved in the survival of cryopreserved mouse ova. Mouse one-cell, two-cell, eight-cell, and blastocyst embryos survived freezing in 1.5 M DMSO in PBS or 1 M glycerol in PBS if cooled at a rate of 0.3°C/min to 1.9°C/min to -80°C before being plunged into liquid nitrogen or liquid oxygen. When the embryos were thawed at relatively slow rates (4° to 25°C/min) and the glycerol or DMSO removed, 50% to 70% of the embryos survived cryopreservation. The transfer of the recovered embryos which were viable resulted in 40% of the embryos developing into newborn mice. These findings have led to the successful cryopreservation of mouse embryos (Table 1), hamster and rabbit embryos (Table 2), rat embryos (Table 3), goat and sheep embryos (Table 4), and cow embryos (Table 5).

The success of these attempts at cryopreservation relied on the fact that the ova and embryos were cooled slowly (<1°C/min) to very low subzero temperatures before storage in liquid nitrogen (-196°C)

TABLE 1

The Cryopreservation of Mouse Ova and Embryos at -196°C by Slow Cooling (<1°C/min) and Slow Thawing (<100°C/min)

Stage of Development	Cryoprotectant	Terminal Temperature[1]	Assay of Viability	Investigator
1 cell to blastocyst	1.5 M DMSO 1.0 M glycerol	-80°C	Development in culture Live birth after transfer	Whittingham et al., 1972; Whittingham et al., 1977c,d
8 cell to blastocyst	2.0 M DMSO	-70°C	Development after culture	Wilmut, 1972
2 to 8 cell embryo	1.0 M DMSO	-50°C to -100°C	Development after culture	Leibo et al., 1974
8 cell embryo	1.0 M DMSO	-110°C	Development after culture Live birth after transfer	Whittingham and Whitten, 1974; Zeilmaker and Verhamme, 1979[2]
Blastocyst	1.5 M DMSO	-80°C	Development after culture Live birth after transfer	Whittingham, 1974
Ova	1.0 M to 2.0 M DMSO	-70°C to -79°C	Normal morphology in vitro fertilization	Tsunoda et al., 1975
Ova	1.5 M DMSO	-80°C	in vitro fertilization Live birth after transfer	Whittingham, 1977b; Parkening et al., 1977
8 cell embryo	1.0 M DMSO	-80°C	Development after culture Pregnancy after transfer	Maurer et al., 1977

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TABLE 1 (continued)

Stage of Development	Cryoprotectant	Terminal Temperature[1]	Assay of Viability	Investigator
8 cell	1.2 M Ethylene Glycol	-79°C	Development after culture Live birth after culture	Miyamoto and Ishibashi, 1977; 1978
Ova	1.5 M DMSO	-79°C	in vitro fertilization Development in culture	Parkening and Chang, 1977

[1]Optimal temperature at which slow cooling was terminated prior to plunging into liquid nitrogen.

[2]Frozen in the oviduct.

TABLE 2

The Cryopreservation of Hamster and Rabbit Ova and Embryos at -190°C by Slow Cooling (<1°C/min) and Slow Thawing (<100°C/min)

Species	Stage of Development	Cryoprotectant	Terminal Temperature	Assay of Viability	Investigator
Hamster	Ova	1.0 M to 2.0 M DMSO	-70°C to -79°C	Normal morphology *in vitro* fertilization	Tsunoda *et al.*, 1975
Hamster	Ova	1.0 M DMSO	-75°C	*in vitro* fertilization	Parkening and Chang, 1977
Hamster	Ova	1.25 M DMSO	-50°C	Normal morphology	Flemming *et al.*, 1979[c]
Hamster	Ova	1.5 M to 2.0 M DMSO	-80°C	Normal morphology Sperm binding to zona pellucida free ova	Quinn *et al.*, 1982
Rabbit	8 cell embryo	1.6 M DMSO	-80°C	Development after culture Pregnancy after transfer	Bank and Maurer, 1974
Rabbit	4 and 8 cell embryo	1.5 M DMSO	-110°C	Development after culture Live birth after transfer	Whittingham and Adams, 1976
Rabbit	Morula	2.0 M DMSO	-100°C	Development after culture Live birth after transfer	Maurer and Haseman, 1976

CONTINUED ON NEXT PAGE....

TABLE 2 (continued)

Species	Stage of Development	Cryoprotectant	Terminal Temperature[1]	Assay of Viability	Investigator
Rabbit	2 cell, 8 cell, 16 cell, and morula	1.5 M DMSO	-100°C	Normal morphology Live birth after transfer	Tsunoda and Sagie, 1977

[1]Optimal temperature at which slow cooling was terminated prior to plunging into liquid nitrogen.

[2]Ova stored at -50°C.

TABLE 3

The Cryopreservation of Rat Ova and Embryos at -196°C After Slow Cooling (<1°C/min) and Slow Thawing (<100°C/min)

Stage of Development	Cryoprotectant	Terminal Temperature[1]	Assay of Viability	Investigator
2,4 and 8 cell	1.5 M DMSO	-80°C	Normal morphology Development after culture Live birth after transfer	Whittingham, 1975
Ovarian oocytes	1.5 M DMSO	-75°C	Normal morphology *in vitro* fertilization	Kasai *et al.*, 1979
8 cell	1.2 to 1.5 M Ethylene glycol	-79°C	Development after culture	Miyamoto and Ishibashi, 1977
8 cell	1.2 M Ethylene glycol	-79°C	Development after culture	Miyamoto and Ishibashi, 1978
Ova	1.5 M DMSO	-75°C	*in vitro* fertilization	Parkening and Chang, 1977

[1]Optimal temperature at which slow cooling was terminated prior to plunging into liquid nitrogen.

TABLE 4

The Cryopreservation of Goat and Sheep Embryos by Slow Cooling (<1°C/min) and Slow Thawing (<100°C/min)

Species	Stage of Development	Cryoprotectant	Terminal Temperature[1]	Assay of Viability	Investigator
Goat	morulae	1.0 M Glycerol or 2.0 M DMSO	-60°C	Development after culture Live birth after transfer	Bilton and Moore, 1976
Sheep	2 cell to morulae	1.5 M DMSO	-65°C	Development after culture[2] Live birth after transfer	Willadsen et al., 1976
Sheep	Morulae to blastocyst	1 M Glycerol or 1-2 M DMSO	not stated	Development in culture	Moore and Bilton, 1976
Sheep	Morulae to blastocyst	1.0-1.5 M DMSO	-30 to -60°C	Normal morphology Development after culture	Willadsen, 1977
Sheep	Day 5 to 8 embryo	1.5 M DMSO	-65°C to -90°C	Development in culture Live birth after transfer	Willadsen et al., 1974

[1]Optimal temperature at which slow cooling was terminated prior to plunging into liquid nitrogen.

[2]Cultured in oviduct of rabbit.

TABLE 5

The Cryopreservation of Cow Embryos in Liquid Nitrogen After Slow Cooling (<1°C/min) and Slow Thawing (<100°C/min)

Stage of Development	Cryoprotectant	Terminal Temperature[1]	Assay of Viability	Investigator
8 cell to blastocyst	1.5 M DMSO	-60°C	Normal morphology Pregnancy after transfer	Wilmut and Rowson, 1973
Blastocysts	2.0 M DMSO	-70°C	Development in culture Live birth after transfer	Wilmut and Rowson, 1973
8-16 cell	1.5 M DMSO	-70°C	Development in culture[2] Live birth after transfer	Wilmut et al., 1975
Morulae to blastocyst	1.5 M DMSO 1.0 M glycerol	-105°C	Development in culture Live birth after transfer	Bilton and Moor, 1977
Blastocyst	1.5 M DMSO	-60°C	Development in culture Live birth after transfer	Lehn-Jensen and Greve, 1972 Trounson et al., 1978a,b Willadsen et al., 1978
Morulae to blastocyst	1.5 M DMSO	-60°C	Development in culture Live birth after transfer	Smoraj et al., 1979

[1]Optimal temperature at which slow cooling was terminated prior to plunging into liquid nitrogen.

[2]Cultured in oviduct of rabbit.

and that recovery of the frozen embryos was accomplished by slow thawing (<100°C/min). Mouse embryos did not survive cooling at a rate greater than 7°C/min (Leibo et al., 1974). However, mouse and sheep embryos did not survive if they underwent slow cooling to above -50°C before being plunged into liquid nitrogen and were recovered by slow thawing (Leibo et al., 1976; Willadsen, 1977). Furthermore, rapid thawing led to a decreased survival of mouse embryos if storage was accomplished by slow cooling to temperatures above -45°C before immersion into liquid nitrogen (Whittingham et al., 1972; Wilmut et al., 1972; Whittingham et al., 1979). Consequently, in 1979, Whittingham et al. observed that a high proportion of viable mouse embryos could be recovered from cryogenic storage by rapid thawing if slow cooling was terminated at a relatively high subzero temperature (-40°C) before storage at -190°C. This gave rise to the two-step freezing system, where ova and embryos could be slowly cooled to high subzero temperatures (-30°C to -40°C), then, rapidly cooled from -30°C and -40°C to -190°C and stored. Recovery could then be accomplished by rapid thawing (>100°C/min) (Wood and Fairand, 1980). Since this discovery, mouse and hamster ova and embryos (Table 6), and sheep and cow ova and embryos (Table 7), have been frozen by the two-step method.

The factors involved in cryopreservation of mammalian ova and embryos are the stage of embryonic development, the freezing medium, the cryoprotectant, the nucleation temperature, the cooling and thawing process, and the assessment of viability after recovery (Leibo and Mazur, 1978; Whittingham, 1980). The principles of each of these factors will be discussed in the following sections. Because of its

TABLE 6

The Two Step Cryopreservation of Mouse and Hamster Ova and Embryos

Species	Stage of Development	Cryoprotectant	Terminal Temperature[1]	Assay of Viability	Investigator
Hamster	Ova	1.5 m-2.0 M DMSO	-40°C	Normal morphology Sperm binding to zona free ova	Quinn et al., 1981
Mouse	8 cell and blastocyst	1.5 M DMSO	-35°C to -50°C	Development in culture Live birth after transfer	Whittingham et al., 1979
Mouse	8 cell to blastocyst	1.5 M DMSO	-20°C to -40°C	Development after culture Live birth after transfer	Wood and Farrart, 1980
Mouse	Morulae	1.5 M to 2 M DMSO	-20°C	Development in culture Live birth after transfer	Kasai et al., 1980
Mouse	8 cell	0.6 M PZ or ?	-30°C	Development in culture Live birth after transfer	Miyamoto and Ishibashi, 1981

[1]Optimal temperature at which slow cooling was terminated prior to plunging into liquid nitrogen.

TABLE 7

The Two-Step Cryopreservation of Sheep and Cow Morulae and Blastocyst[1]

Species	Cryoprotectant	Terminal Temperature[2]	Investigator
Sheep	1.5 M DMSO	-30°C to -48°C	Willadsen, 1977
Cow	1.5 M DMSO	-30°C to -37°C	Willadsen et al., 1978b
Cow	1.5 M DMSO	-33°C	Massip et al., 1979 Teruit and Eldsen, 1980
Cow	1.5 M DMSO or 1.0 M Glycerol	-30°C to -40°C	Lehn-Jensen, 1980
Cow	1.4 M Glycerol	-30°C to -40°C	Lehn-Jensen et al., 1981; 1983 Bouyssou and Chapin, 1982b Lehn-Jensen and Greve, 1982
Cow	1.2 M Propanediol	not stated	Renard et al., 1981a
Cow	1.5 M DMSO or 1.4 M Glycerol	-30°C	Bouyssou and Chapin, 1982a
Cow	1.5 M DMSO	-33°C to -43°C	Farrand et al., 1982 Santos et al., 1981

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TABLE 7 (continued)

Species	Cryoprotectant	Terminal Temperature[2]	Investigator
Cow	1.5 M Ethylene Glycol 1.35 M Glycerol 1.3 M Glycerol:DMSO (50:50)	-35°C	Eldsen et al., 1982

[1]The normality of the frozen-thawed embryos were judged by the ability to develop in culture and/or the production of pregnancy and live birth after transfer.

[2]Optimal temperature at which slow cooling was terminated and embryos were immersed into liquid nitrogen.

ultimate importance, the cooling and thawing process will be discussed first.

Cooling and Thawing Rates

The proper cooling and thawing rates are crucial for embryo survival during cryopreservation.

The rates at which embryos can be cooled and thawed have previously been discussed. The rationale of why the embryo is restricted to slow cooling rates requires an understanding of the physical events that occur during the embryo's exposure to subzero temperatures. As a cell is cooled in medium in which ice formation has occurred, more ice forms as the temperature is lowered. As ice formation increases the concentration of solute particles in the fluid surrounding the cell increases. The increase in solute causes an osmotic force upon the cell and cellular dehydration occurs (Mazur, 1970; Leibo, 1979). Cellular dehydration causes cellular shrinkage. This phenomena has been observed by the aid of a cryoscope (Leibo, 1977). Injury to cells during freezing can be caused by both supraoptimal and suboptimal freezing rates (Mazur, 1965; 1977a).

Freezing of cells at supraoptimal rates causes inadequate dehydration of the cells. This allows the formation of intracellular ice (Mazur, 1965; 1977a,b). Intracellular ice formation in mouse ova has been correlated with ovum death when cells were cooled too fast during cryopreservation (Leibo *et al.*, 1978; Rall *et al.*, 1983).

Suboptimal freezing rates have been defined as rates in which the intracellular water remains in equilibrium with the extracellular

water and ice (Mazur, 1977a,b). The damage to the ovum during sub-optimal freezing could be the result of the removal of water as ice, the concentrating of solutes of high and low molecular weight, the shrinkage of the cell, or the precipitation of solutes. The damage done to cells during slow freezing is termed a solution effect. Solution effects in mammalian ovum cryopreservation are prevented by the addition of glycerol, DMSO, or other permeating compounds which function to prevent the concentrating of solutes (Mazur, 1970).

Mouse embryos in the presence of a cryoprotectant survive rapid cooling to -196°C only if they are slow cooled to -50°C prior to being plunged into liquid nitrogen. Embryos, which are frozen by such a method only survive cryopreservation if they are slowly thawed (<100°C/min). Damage incurred by rapid thawing of embryos occurs between -70°C and -20°C. The lethality of rapid thawing in such a system results from the rapid change in solute concentration as the cryoprotectant and medium components melt. Slow thawing allows sufficient equilibration time of solutions as the medium is warmed (Leibo, 1974).

Mammalian ova and embryos can survive rapid thawing if slow cooling is terminated at high subzero temperatures and the ova and embryos are rapidly cooled to -196°C (Tables 6 and 7). Observation of mouse embryos cooled slowly to -40°C, followed by rapid cooling to -196°C, and subsequent slow thawing revealed the lethality of the slow thawing. No intracellular ice was formed in slow cooling of ova by such a method. But upon slow thawing, intracellular ice formed in the ova at -80°C and ice crystallization spread beyond the boundary of the cell plasma membranes at -55°C. This spreading of ice crystallization

was determined to be the lethal factor when slow thawing was employed. Ice formation was not observed with rapid thawing (Rall et al., 1980).

In summary, successful cryopreservation of mammalian embryos can occur only at optimal combinations of cooling and thawing rates. Slow cooling to low subzero temperatures must be followed by slow thawing and slow cooling to high subzero temperatures must be followed by rapid thawing after storage at -196°C to yield viable embryos. The proper cooling and thawing rates allow for the proper exchange of water and solutes between the intracellular and the extracellular compartments. This exchange prevents damage due to intracellular ice formation or solution effects.

Stage of Embryonic Development

The stage of embryonic development does not appear important in the cryopreservation of mice, rabbit, rat, and sheep embryos, as can be seen in Tables 1 through 4. Unfertilized ova and embryos from the one-cell stage to the blastocyst stage of development can be successfully cryopreserved with no apparent differences among stages of development. However, the preimplantation embryos of other species are more labile during cryopreservation. Pig embryos from eight-cell stage to blastocyst stage of development cannot survive cooling to 0°C (Polge et al., 1974). Furthermore, the cryopreservation of cattle embryos is dependent upon the stage of development. Early cleavage stages of cow embryos, 8-cell to 16-cell stages (Wilmut et al., 1975) and 8-cell to early morulae (Trounson et al., 1976a) are damaged by cooling to 0°C. The later stages of development of cattle preimplantation embryos, morulae to blastocysts, survive cooling to 0°C

(Trounson et al., 1976a,b) and 4°C (Bon Durond et al., 1981, 1982; Linder et al., 1982) without a decrease in viability.

The presence of the zona pellucida around the preimplantation embryo has been speculated as an important factor in cryopreservation of mouse embryos (Wilmut, 1972). Hatched bovine blastocysts, freed of their zona pellucida, as well as, blastocysts retaining their zonae pellucidae, did not survive cryopreservation (Trounson et al., 1978). The viability of the zona pellucida-free bovine blastocysts may be due to the stage of development of the embryo rather than the lack of any protective action stemming from the zona pellucida. Hamster ova, which have had their zona pellucida enzymatically removed, have survived cryopreservation at a similar rate as the zona pellucida intact ova (Flemming et al., 1979).

Freezing Medium

Mammalian embryos were first successfully cryopreserved by Whittingham et al. (1972) who used Dulbecco's phosphate buffered saline (PBS) to which a cryoprotectant, DMSO, had been added. Since this report, PBS has been the medium of choice for cryopreservation (Whittingham, 1980). A modification of PBS (PBS supplemented with 0.33 mM pyruvate, 5.56 mM glucose, and 3 mg/ml bovine serum albumin) has, also, been used in the cryopreservation of mammalian embryos (Whittingham and Adams, 1976; Flemming et al., 1979; Wood and Farrot, 1980). The addition of 10% fetal calf serum (Massip et al., 1979), 10% sheep serum (Willadsen et al., 1978), and polyvinylalcohol (Creighton and Lindo, 1983) to PBS does not decrease embryo viability

after cryopreservation. The substitution of PBS with Hams F-10 medium did not give any significant difference in the viability of bovine embryos after freezing and thawing. But the development of the thawed embryos in culture was impeded by the Hams F-10. Zona pellucida-free hamster ova, however, showed a preference for cryopreservation in HEPES buffered Tyrode's solution. Viability of hamster ova was not different after cryopreservation but human sperm did not bind as well to the ova frozen in PBS as to those ova frozen in the modified Tyrode's solution (Quinn et al., 1982).

The purpose of the freezing medium is to maintain the ova and embryos during handling. Therefore, a medium that supports the development of the embryo is suitable as a medium for cryopreservation of the embryo (Whittingham, 1980). No medium has been found to be incompatible with the cryopreservation procedure.

Cryoprotectants

Cryoprotectants are compounds which offer protection to organisms as they are exposed to extremely low temperatures. The cryoprotectants can be grouped into two categories: those that penetrate the cell and those that do not penetrate the cell. The former group offers protecttion from osmotic damage caused by slow cooling. This is accomplished by preventing the concentrating of the extracellular solutes during cooling and, thus, preventing cellular dehydration. The nonpenetrating group of cryoprotectants prevent intracellular ice damage caused by rapid cooling. This is accomplished by the creation of osmotic forces which cause cellular dehydration (Merryman, 1963). The exact mechanisms of action of the cryoprotectants are not known.

The cryoprotectants which have been helpful in the cryopreservation of mammalian ova and embryos are summarized in Tables 1 through 7. They are all penetrating agents, protecting against slow cooling damage. Nonpenetrating agents, such as sucrose and polyvinylpyrolidine do not protect mammalian embryos during cryopreservation (Wilmut, 1972; Wilmut et al., 1973). This is probably due to the utilization of slow cooling for cryopreservation.

Nucleation Temperature

The temperature at which extracellular ice formation is induced in the freezing medium as the temperature of the medium is being lowered is the nucleation or seeding temperature. Nucleation is usually induced at 1°C to 2°C below the freezing point of the medium (Whittingham, 1977). Nucleation can be accomplished in various ways: (1) touching the surface of the suspending medium with ice crystals contained in a Pasteur pipette or wire cooled in liquid nitrogen, (2) touching the outside of the freezing container with dry ice or a cold metal bar, or (3) tapping the samples (Whittingham, 1980). If nucleation is not accomplished the suspending medium may supercool and ice formation will occur spontaneously at a much reduced temperature (Whittingham, 1977).

Supercooling of the medium has been shown to be detrimental to the survival of mammalian embryos during cryopreservation. Neglecting the nucleation of the suspending medium led to a decrease in survival of sheep embryos (Willadsen, 1977). Furthermore, seeding at temperatures below normal, -10°C and -12°C, was lethal to mouse embryos (Miyamoto and Ishibashi, 1981; Whittingham, 1977).

Spontaneous ice formation of supercooled fluids causes a rise in temperature of the fluid due to the released latent heat during the phase change. In the case of supercooling during cryopreservation, the suspending medium temperature is elevated during spontaneous ice formation. This rise in the temperature of the freezing medium occurs while the external temperature is being lowered. The thermal equilibration of the freezing medium with the external temperature causes the embryos to be cooled at rates much faster than that observed. This increase in cooling rate could lead to decreased survival of the embryos. Adjustment of the cooling rate during spontaneous nucleation, however, does not explain the total decrease in embryo survival. The process of supercooling may have altered the permeability of oocyte plasma membrane which would also lead to a decrease in survival (Whittingham, 1977).

Assessment of Viability

The assessment of the viability of mammalian embryos after freezing and thawing can be made by several methods: (1) morphological observation, (2) use of vital dyes, (3) histological observations, (4) metabolic assessment, (5) fertilization of oocytes, (6) development *in vitro*, (7) development after xenogenous culture, and, (8) transfer of the embryo to recipient females (Whittingham, 1978b). The techniques used to assess viability for cryopreserved mammalian ova and embryos are summarized in Tables 1 through 7. All the assays of viability have their validity, but the ultimate purpose of the cryopreservation of mammalian ova and embryos is the fertilization and subsequent transfer of the embryos to yield live offspring.

Successful cryopreservation of unfertilized ova has been accomplished in the mouse (Tsumoda et al., 1975; Whittingham, 1977; Parkening and Chang, 1977), hamster (Tsunoda et al., 1975; Parkening and Chang, 1977; Flemming et al., 1979; Quinn et al., 1982), and rat (Kasai et al., 1979; Parkening and Chang, 1977). In vitro fertilization of cryopreserved ova has been reported. Little has been done to compare the fertilization rate of these cryopreserved ova with nonfrozen controls. However, Flemming et al. (1979) has reported that cryopreserved zona pellucida-free hamster ova bind homologous and heterologous sperm at the same rate as the controls.

Transfer of frozen-thawed mouse embryos (Whittingham and Anderson, 1976), rabbit embryos (Maurer and Haseman, 1976; Whittingham and Adams, 1976), and cow embryos (Tables 5 and 7) has yielded pregnancy rates lower than nonfrozen controls. Ultrastructural examination of cryopreserved mouse embryos shows no morphological explanation for this phenomenon. The metabolic processes of mouse embryos may have been slowed by cryopreservation because culture of cryopreserved mouse embryos for 24 hours prior to transfer increased the pregnancy rates compared to that of nonfrozen controls (Whittingham and Anderson, 1976). Culture of cow embryos after cryopreservation was not beneficial to their transfer (Willadsen et al., 1978; Renard et al., 1981).

Xenogenous Fertilization

The oviduct of the pseudopregnant rabbit has been shown to support the development of the embryos of many different mammalian species. A list of species whose embryos have been shown to develop

in the rabbit oviduct can be found in Table 8. Umbaugh in 1949 demonstrated that the rabbit oviduct can support not only the development of mammalian embryos, but also the fertilization of bovine ova. Those species whose ova have been fertilized in rabbit oviduct can be found in Table 9. The rabbit is not the only species which can support fertilization of heterologous gametes in its oviduct. The oviduct of the ewe (Sreenan, 1970) and of the gilt (Shea et al., 1976; Bedirian et al., 1975) can support the fertilization of bovine embryos. Furthermore, the mouse oviduct can support the fertilization of hamster ova while the hamster oviduct can support the fertilization of mouse and rabbit ova. Finally, the rat oviduct can support the fertilization of mouse and hamster gametes (Saling and Bedford, 1981). This fertilization of ova of one species in the oviduct of another species is termed xenogenous fertilization.

TABLE 8

The Culture of Mammalian Ova in the Rabbit Oviduct

Embryo Donor	Reported Development	Investigator
Mouse	2 cell to Blastocyst	Brinster and Ten Broek, 1969
Rat	Early Blastocyst to Hatched Blastocyst	Yoshinaga and Adams, 1967
Ferret	2-4 cell to 8-12 cell Blastocyst to Expanded Blastocysts	Chang, 1966 Chang et al., 1971
Snowshoe Hare	2 cell to Blastocyst	Chang, 1965
Bovine	Early Cleavage to Blastocyst	Sreenan and Scanlon, 1968 Hafez and Sugie, 1963 Lawson et al., 1972 Sreenan et al., 1968 Adams et al., 1968
Ovine	Early Cleavage to Blastocyst	Averill et al., 1955 Adams et al., 1961 Hunter et al., 1962 Adams et al., 1968 Lawson et al., 1972
Equine	32 cell to Hatched blastocyst	Allen et al., 1976

TABLE 9

Xenogenous Fertilization of Mammalian Ova in the Rabbit Oviduct

Species	Investigators
Mouse	Saline and Bedford, 1981
Hamster	Demayo *et al.*, 1980
Pig	Hirst *et al.*, 1981
Cow	Umbaugh, 1949 Trounson *et al.*, 1977 Sreenan, 1970 Hirst *et al.*, 1981
Squirrel Monkey	Demayo *et al.*, 1980
Baboon	Kuehl, 1983

MATERIALS AND METHODS

Animal Care

Squirrel monkeys of the Bolivian and Guyanan types were obtained from Primate Imports Corp. (Port Washington, NY) and South American Primates (Miami, FL), respectively. They were housed in groups of six, in stainless steel, flush-type, cages from October through June. During the summer, June through September, they were housed outdoors, in groups of 50, in 4 outdoor colonies (Jarosz and Dukelow, 1976). The animals were fed High Protein Monkey Chow^{R}, Jumbo 5047 (Ralston Purina Co., St. Louis, MO), water ad libitum and fruit as a diet supplement. While being housed indoors they were exposed to 12h:12h light:dark (0600 hrs to 1800 hrs light) cycle using fluorescent lighting.

The golden hamsters were obtained from the State of Michigan Health Laboratories. The hamsters were housed in groups of six in plastic cages with stainless steel tops with ground corn cobs as bedding (San-I-Cell^{R}, Paxton Processing Co., Paxton, IL). Wayne Laboratory Animal Diet (Allied Mills Inc., Chicago, IL) and water was fed ad libitum. The animals were exposed to a light/dark cycle of 14:10 hrs (0600 hr to 1800 hr lights on).

Rabbits were housed singly in stainless steel cages with ground corn cobs as bedding. The rabbits were fed with Laboratory Rabbit

Chow^{R} (Ralston Purina Co.) and water ad libitum. Rabbits were not maintained under a specific light/dark cycle.

Cryopreservation

The procedure for cryopreservation consists of: ovum collection, addition of the cryoprotectant, nucleation of the medium, slow cooling, thawing, removal of the cryoprotectant and assessment of the ovum's viability.

Medium: The media used to supply a safe environment for the hamster ovum during cryopreservation was either Dulbeccos phosphate buffered saline (PBS) (Gibco Laboratories, Grand Island, NY) or a modification of the squirrel monkey in vitro fertilization medium used by Kuehl and Dukelow (1979), TC-199. The basic contents for this medium are listed in Table 10. In order to facilitate the removal of cumulous cells from the hamster ova during ovum collection, the collection medium was supplemented with 1 mg/ml hyaluronidase (Sigma Corp., St. Louis, MO). Squirrel monkey ova were manipulated in the medium as described in Table 10. All media were sterilized by filtration through a 0.45 μm Millipore filter and stored in a sterile Vacutainer at 4°C.

Ovum Collection: Hamster ova were obtained from oviducts of animals superovulated with an intraperitoneal injection of 30 IU pregnant mare serum (PMS, Folligon^{R}; Intervet Laboratories; Bar Hill, Cambridge, Great Britain) between 0900 hrs and 1200 hrs followed 56 to 64 hrs later with an I.P. injection of 30 IU human chorionic gonadotropin, HCG (APL^{R}; Ayerst Laboratories Inc., NY) (Yanagimachi and

TABLE 10

The Composition of the Supplemented TC-199 Medium used for Gamete Manipulation

Ingredient	Amount	Source
TC-199[1]	80%	GIBCO Laboratories Grand Island, N.Y.
GG Free Fetal Bovine Serum[2]	20%	GIBCO Laboratories Grand Island, N.Y.
Sodium Pyruvate	115.2 µg/ml	Sigma Chemical Co. St. Louis, MO
Gentamicin	0.1 mg/ml	Schering Corp. Kenilworth, N.J.
Penicillin-Streptomycin	100 units and 100 µg/ml	North American Biological Miami, FL
Hyalarunidase[3]	1 mg/ml	Sigma Chemical Co. St. Louis, MO
Heparin[4]	1 unit/ml	The Upjohn Co. Kalamazoo, MI

[1]Medium 199 with 25 mM HEPES buffer, Earle's salts and L-Glutamine.

[2]Heated at 56°C for 30 minutes.

[3]Added when collecting hamster ova to remove camalus cells.

[4]Added to prevent blood clotting when collecting monkey ova.

Chang, 1964). These hamsters were sacrificed, by cervical dislocation, 14 to 16 hrs after HCG administration. The oviducts were removed, dissected free of fat and flushed from the fimbrie with 0.3 ml of medium. Mature ova were counted and held in an ice bath until preservation.

Squirrel monkey ova were collected by laparoscopic aspiration of follicles (Dukelow and Ariga, 1976) from females after administering a regimen of gonadotropins to induce ovulation (Dukelow, 1970). This ovulatory regime of gonadotropins consisted of injecting I.M. for four days 1 mg follicle stimulating hormone, FSH (Bruns Biotec, Oakland, CA) with an intramuscular injection of 250 I.U. HCG on the fourth day (Dukelow, 1979). During the summer months, July to October, five days of I.M. injections of 1 mg FSH were administered due to the seasonality of the squirrel monkey (Harrison and Dukelow, 1973). The squirrel monkeys were then laparoscoped 12 to 18 hrs after the administration of HCG. Follicles were then aspirated with a 25 gauge, 5/8" needle into 0.1 ml medium. The follicular contents were deposited into an 8 chamber tissue culture chamber slide (Lab-Tek, Miles Laboratories Inc., Naperville, IL) and incubated in a 37°C, moist atmosphere with 5% CO_2 in air until cryopreservation.

Cryoprotectant: The cryoprotectant used in this study was dimethylsulfoxide (DMSO; Baker Chemical Co., Phillipsburg, PA). A stock solution of 3 M or 4 M DMSO was dissolved in either Dulbecco's phosphate buffered saline or supplemented TC-199 and was stored at 4°C.

Squirrel monkey or hamster ova were placed in 0.1 ml of media in a 2 ml (1 cm x 7 cm) glass ampule (Wheaton Scientific, Millville, NJ). The ampules containing hamster ova were then placed in an ice bath while those ampules containing squirrel monkey ova were placed in an ice bath or held at room temperature. An equal volume (0.1 ml) of either 3 M or 4 M DMSO was added, in one step, to the ampules to bring the final concentration of DMSO to either 1.5 M or 2 M. The DMSO was added at the same temperature as that of the medium containing the ova. In one trial monkey ova were placed directly in 0.2 ml of 3 M DMSO. After the addition of DMSO the ampules were sealed using a methane-oxygen hand sealing machine, Model HSI (Cozzoli Machine Co., Plainfield, NJ). The ova were now ready for freezing.

Nucleation of the Medium: The sealed ampules, containing ova, were placed in a 95% ethanol bath which had been cooled to a temperature of -5°C to -7°C. Nucleation or seeding was accomplished by touching the side of the ampule with forceps which had been immersed in liquid nitrogen. Once ice formation was initiated the ampules were placed in the cooling apparatus.

Slow Cooling: The apparatus used for slow cooling is shown in Figure 1. An unsilvered 900 ml dewar flask (9 cm x 30 cm) (Ace Glass Inc., Vineland, NJ), was filled with 350 ml of 95% ethanol. This dewar flask was of 6 Torr vacuum. The unsilvered dewar flask was placed in a 4.5 liter silvered dewar flask which had been filled with liquid nitrogen. The cooling rate of the ethanol bath was determined by the least squares regression analysis of the decline in the temperature of the bath with time. The cooling rate was a function of:

Figure 1. The slow cooling apparatus used in the cryopreservation of hamster and squirrel monkey ova.

the volume of ethanol in the unsilvered dewar flask, the depth of immersion of the usilvered dewar flask in the liquid nitrogen bath and the vacuum of the unsilvered flask. The depth of immersion of the unsilvered dewar flask in the liquid nitrogen bath was regulated by a laboratory jack. The cooling rates achieved for the cooling apparatus ranged from 0.19°C/min to 0.35°C/min.

Ampules containing hamster ova, which had been seeded, were placed in the cooling apparatus at temperatures of -10°C, -20°C or 30°C. The ampules were removed from the cooling apparatus and plunged in liquid nitrogen after they had been cooled to either -40°C, -50°C, -60°C, -70°C or -80°C. This 3x5 factorial was used to determine the optimum temperature range for slow cooling of hamster ova. Ampules containing squirrel monkey ova were placed in the cooling apparatus at the seeding temperature and cooled to -60 or -80°C before plunging into liquid nitrogen. The storage time for the ova ranged from 1 week to 3 months

Thawing: There were three methods used to thaw the cold stored ampules, tested:

1) Ampules were placed in a 600 ml beaker filled with 200 ml of 95% ethanol which had been cooled to -110°C. The ethanol bath was then allowed to equilibrate with room temperature. Least squares regression analysis of the change in temperature of the ethanol bath showed a warming rate of 1 to 4°C/min.

2) Ampules were placed in a styrofoam cup and allowed to warm. Observation of the time required for melting of the contents of the ampule gave an estimate of warming rates of 17.6°C/min - 27.6°C/min.

3) Ampules were placed in an ice bath. A warming rate of 92°C/min was estimated by the same method previously given.

Removal of the Cryoprotectant: Once the contents of the ampule had thawed the DMSO was removed by seria dilution at 4°C. This was accomplished by the addition of 0.2 ml, 0.2 ml, 0.4 ml and 1.0 ml of freezing medium at 1 minute intervals.

Assessment of Viability: Viability of cryopreserved ova was assessed by: morphological normality, the ovum's ability to exclude trypan blue, and the ovum's ability to incorporate fluorescein diacetate (FDA).

The criteria for morphological normality was the appearance of a light, nongranular and translucent vitellus (Quinn et al., 1982) and the presence of an intact zona pellucida. This subjective analysis of viability led to the need for a more objective determination of viability. Hamster ova were incubated in 25 µl of 0.2% trypan blue for 1 min and washed in 25 µl of medium. If the ooplasm was free of trypan blue the ova was judged viable. Squirrel monkey ova were placed in a depression slide containing 25 µl of 15 µM FDA in PBS. After holding the ova for 1 min, the ova were examined under a fluorescence microscope (Leitz BG12 and BG38 exciter filter and a K510 long pass barrier filter (The Microscope Co., New Castle, PA). If fluorescence was observed the ova was classified as viable.

Xenogenous Fertilization Procedure

The procedure for xenogenous fertilization consists of: ovum collection, sperm collection, deposition of gametes in the rabbit

oviduct and embryo recovery. Ovum collection has been discussed in a previous section.

Sperm Collection: Hamster sperm was collected from the epididymi of mature males. Hamsters were sacrificed, by cervical dislocation, and their epididymi were dissected. The cauda epididymus was minced in 1 ml of medium. A 0.05 ml aliquot of this solution was then diluted five times and a sample of this solution was evaluated under the light microscope for motility and structural normality.

Semen, obtained by electroejaculation of unanesthetized male squirrel monkeys, was collected in 0.5 ml of medium. A sample of this solution was evaluated for motility and structural normality under the light microscope.

Hamster and squirrel monkey sperm suspensions were held in a 37°C water bath until deposited in a rabbit oviduct within 1/2 to 1 hour. Twenty-four hours after collection, the sperm concentration was determined with a hemocytometer.

Rabbit Surgery: Adult female rabbits (New Zealand White) were given 100 I.U. HCG, I.V., to induce pseudopregnancy (Harper, 1963). On the day of surgery, each rabbit was anesthetized with 60 mg/2.25 kg body weight of sodium pentobarbital (Nembutal^{R}; Abbott Laboratories, North Chicago, IL) followed by ether inhalation to maintain a surgical plane of anesthesia. The reproductive tract was then exposed through a 7 cm mid-ventral incision. Ova, in 5 µl aliquots were deposited using a Micropetter^{R} (SMI, Scientific Manufacturing Industries, Emeryville, CA) into the fimbriated end of the ampulla. Depending on the number of ova, 1 to 4 aliquots were deposited. Viable frozen

thawed ova were placed in one oviduct and nonfrozen control ova were placed in the opposite oviduct.

After deposition of ova 0.05 ml of nonspecific sperm solution was deposited. Sperm were deposited using a 0.25 ml tuberculin syringe, fitted with a 20 gauge, 1.5" needle to which 5 cm polystyrene tubing (0.034 inches id, 0.050 inches od; Clay Adams, Parsippany, NJ) was affixed. After insemination the uterotubal junction was ligated with 00 gut suture and the abdominal incision closed.

Embryo Recovery: Each rabbit was killed by cervical dislocation 24 hrs later and the reproductive tract was removed. Using a 5ml syringe with blunted 25 gauge, 5/8" needle, medium (2 ml) and air (1.5 ml) was flushed through the oviduct from the uterine end. The oviductal contents were collected in a watch glass and under a dissecting microscope recovered embryos were observed. Fertilization was judged to have occurred if 2 polar bodies and 2 pronuclei were observed or if cleavage had occurred.

Statistical Analysis

All percentage data, analyzed by parametric statistical analysis, was tranformed using the arc sine $\sqrt{X}$ transformation. The effects of medium, concentration of DMSO and warming rate on the viability of cryopreserved hamster ova were analyzed using the Student's t-test. The optimum temperature range for slow cooling of hamster ova was analyzed by 2-way analysis of variance.

Parametric statistical analysis was not used on the analysis of factors affecting the cryopreservation of squirrel monkey ova. The

low number of monkey ova in each ampule prevented the assumption of continuity of the percentage data needed for parametric statistical analysis. Chi square analysis and Fishers Exact test was used to analyze the squirrel monkey data (Zar, 1974).

RESULTS

A total of 4,908 hamster ova were cooled to -196°C and stored for 1 day to 3 months. After thawing, 3,267 ova (66.6%) were recovered and 2,842 (87.0%) of the recovered ova were judged to be viable by their ability to exclude trypan blue. A total of 363 squirrel monkey ova were frozen and 342 (94.2%) ova were reocvered after storage. Of the recovered ova, 146 (42.7%) were judged viable by their ability to incorporate fluorescein diacetate.

Hamster ova were cooled at rates of 0.19-0.25°C/min to a temperature of -80°C. The ova were then plunged into liquid nitrogen and stored. Recovery of these ova was accomplished by slow warming at rates of 1 to 4°C/min. The effects of storage medium, PBS vs. TC-199 on the cryopreservation of hamster ova is shown in Figure 2. No significant difference was observed on the viability of hamster ova cryopreserved in PBS or TC-199 (t_3 = 1.53) as judged by the ovum's exclusion of trypan blue. The ova were then cultured for 3 hr at 37°C in a 5% CO_2, in air atmosphere with 100% humidity. As can be seen from Figure 2, no significant difference was observed, after 3 hr culture, in the viability of the hamster ova with the two media (t_3 = 0.76). Since TC-199 is the medium used in the xenogenous fertilization of hamster and squirrel monkey gametes, this medium was utilized in all future cryopreservation trials.

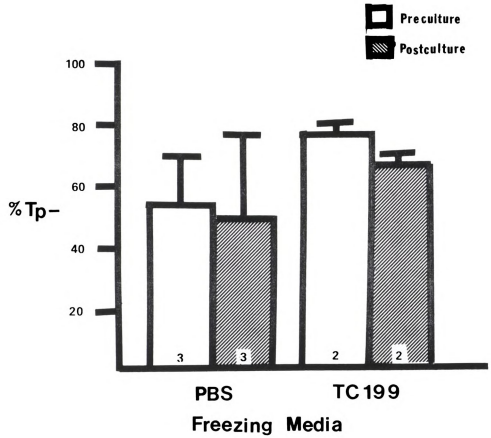

Figure 2. The viability of hamster ova after cryopreservation in 1.5 M DMSO-PBS or 1.5 M DMSO-TC199, immediately after recovery by slow thawing and after culture for 3 hrs. Bars indicate standard error. The number in the data bars indicates the number of trials.

Thawing of hamster ova at 1-2°C/min or 92°C/min had no significant effect on the viability of cryopreserved hamster ova (t_2 = 1.56) and no difference was observed after the culture of the ova for 3 hrs (t_2 = 3.79). These results are shown in Figure 3.

Increasing the concentration of DMSO from 1.5 M to 2.0 M had no significant effect on the viability of cryopreserved hamster ova immediately after thawing (t_8 = 1.32). No difference in viability was observed with these two treatments after a 3 hour culture of the ova (t_8 = 1.60). These results are shown in Figure 4.

The temperature range for which hamster ova must be slow cooled, to yield maximum viability, was investigated. Hamster ova were rapidly cooled to -10°C, -20°C or -30°C after the seeding of the ampules. The ampules were then slow cooled to -40°C, -50°C, -60°C, -70°C or -80°C before they were plunged into liquid nitrogen. The results of this 3x5 factorial experiment are shown in Figure 5. The analysis of variance of this data, as shown in Table 11, shows no significant effect caused by the temperature at which slow cooling is initiated and no significant effect caused by the temperature at which slow cooling is terminated. There was no interaction between the two main effects.

Squirrel monkey ova were cooled slowly to -80°C in 1.5 M DMSO-TC-199 and stored in liquid nitrogen. Survival upon thawing was significantly decreased when compared to nonfrozen controls (χ^2_2 = 70.6) as shown in Figure 6. When the concentration of DMSO was increased to 2 M there was a significant increase in the viability of squirrel monkey ova when compared to ova frozen in 1.5 M DMSO (χ^2_2 = 10.1), as is shown in Figure 6.

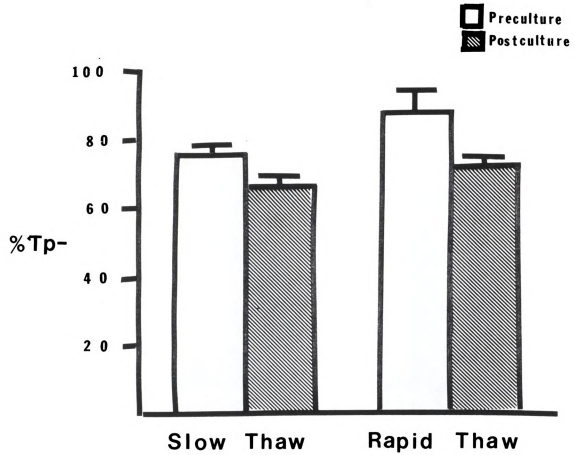

Figure 3. The viability of hamster ova after the cryopreservation in 1.5 M DMSO-TC199 and recovered by slow thawing (1-2°C/min) or rapid thawing (≈92°C/min). Bars indicate standard error.

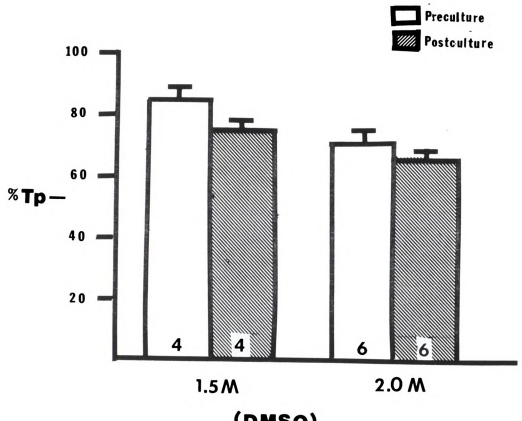

Figure 4. The viability of hamster ova after cryopreservation in 1.5 M DMSO-TC199 or 2.0 M DMSO-TC199 immediately after recovery and after 3 hrs in culture. Bars indicate standard error. The number in the data bars indicates the number of trials.

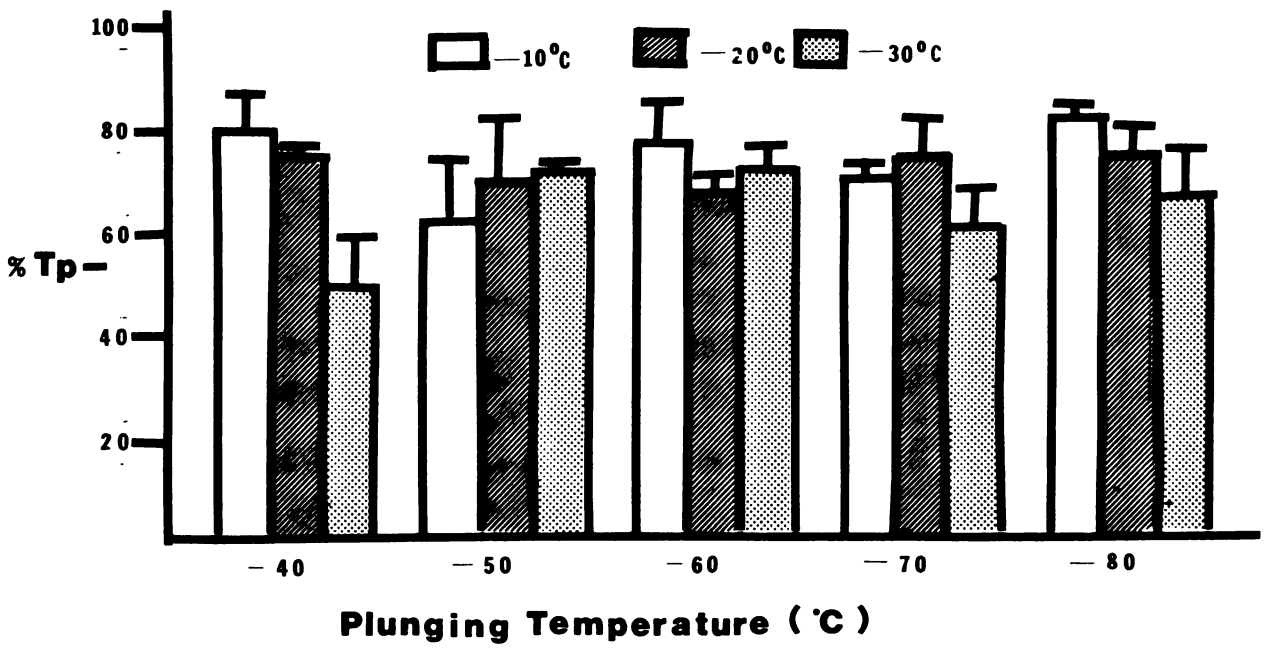

Figure 5. The viability of hamster ova rapidly cooled to -10°C, -20°C, or -30°C and slow cooled to -40°C, -50°C, -60°C, -70°C or -80°C before plunging into liquid nitrogen. Recovery was accomplished by rapid thawing at ≈92°C/min.

TABLE 11

The Analysis of Variance of the Effects of the Initial Slow Cooling Temperature and the Final Slow Cooling Temperature on the Viability of Frozen Thawed Hamster Ova

Source of Error	Degrees of Freedom	Sum of Squares	Mean Square Error	F Statistic
Initial Temperature	2	167.2	83.6	$F_{2,15} = 2.34$
Plunging Temperature	4	111.7	27.9	$F_{4,15} = 0.78$
Interaction	8	406.9	50.9	$F_{8,15} = 1.42$
Residual	15	535.9	35.7	
Total	29	1221.7		

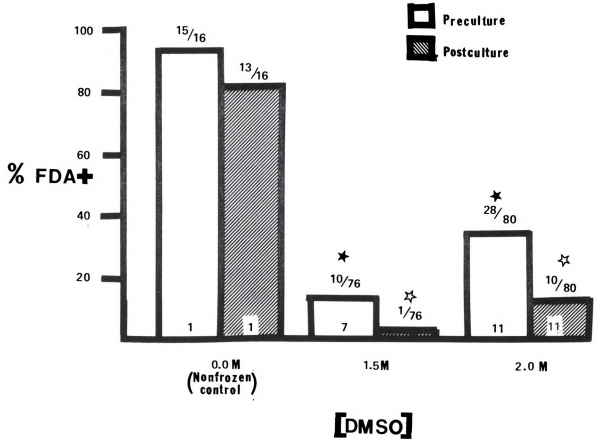

Figure 6. The viability of squirrel monkey ova cryopreserved in 1.5 M DMSO-TC199 or 2.0 M DMSO-TC199, immediately upon recovery and after 3 hrs in culture. The number in the data bars indicates the number of viable monkey ova per recovered ova. The closed stars indicate a significant difference from the preculture nonfrozen controls. The open starts indicate a significant difference from the postculture nonfrozen controls.

Preliminary trials indicated that the decrease in viability after the cryopreservation of squirrel monkey ova may be due to the temperature at which DMSO is added, 0°C. The addition of DMSO at room temperature, 20°C, significantly increased the viability of squirrel monkey ova when frozen in 1.5 M and 2 M DMSO. When compared to similar concentrations of DMSO added at 0°C, Figure 7 (χ_1^2 = 25.5 and 8.4, respectively). This increase in viability was seen immediately after recovery and after the recovered ova were cultured for 3 hours (χ_1^2 = 2.0 and 8.7, respectively) as seen in Figure 8. DMSO was added to squirrel monkey ova at 20°C, prior to slow cooling, in all the following trials.

DMSO, when used as a cryoprotectant, at concentrations of 1.5 M, 2 M or 3 M was equally effective at producing viable squirrel monkey ova. No significant effect, due to these concentrations of DMSO, was observed immediately after thawing (χ_2^2 = 0.4) or after the recovered ova were cultured for 3 hours (χ_2^2 = 1.1). These results are shown in Figure 9. (Data for 1.5 M and 2.0 M DMSO were taken from Figure 8.)

Thawing rates of 1-2°C/min, 17.6-27.6°C/min or <92°C/min showed no significant effect on the viability of squirrel monkey ova after freezing. No difference was observed immediately upon recovery (χ_2^2 = 2.7) or after the ova were cultured for 3 hours (χ_2^2 = 2.8). These results are shown in Figure 10.

The fertility of the frozen hamster and squirrel monkey ova is shown in Table 12. Hamster ova, when frozen under optimal conditions, showed a significant decrease (χ_2^2 = 13.1) in their ability to be

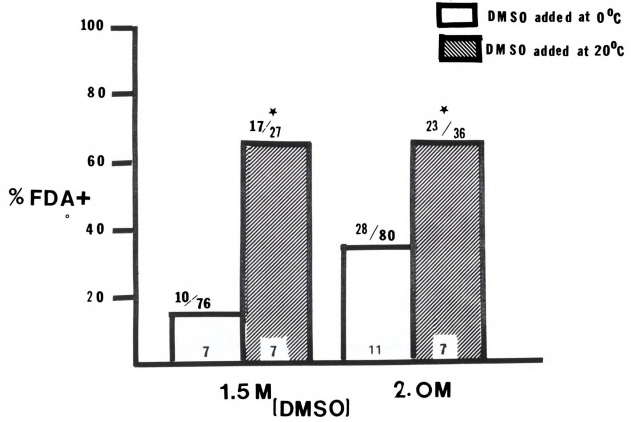

Figure 7. The effect of the temperature of the addition of DMSO on the viability of cryopreserved squirrel monkey ova. The viability was determined immediately upon recovery of the ova. The number in the data bars indicates the number of trials. The fractions above the data bars indicate the number of viable ova per recovered ova. The stars indicate a significant difference between the temperatures of the addition of DMSO, for the same concentration of DMSO.

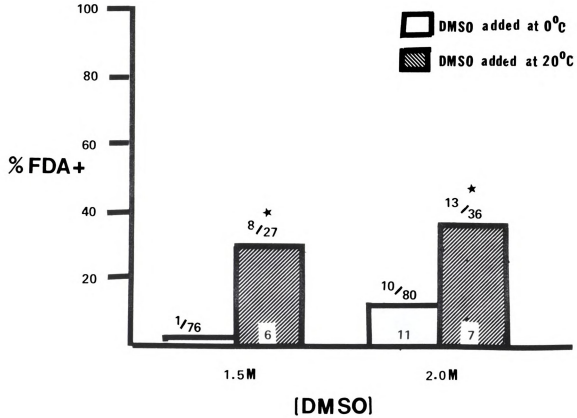

Figure 8. The effect of the temperature of the addition of DMSO on the viability of cryopreserved squirrel monkey ova. The viability was determined after the recovered ova were cultured for 3 hrs. The number in the data bars indicates the number of trials. The fractions above the data bars indicate the number of viable ova per recovered ova. The stars indicate a significant difference between the temperatures of the addition of DMSO, for the same concentration of DMSO.

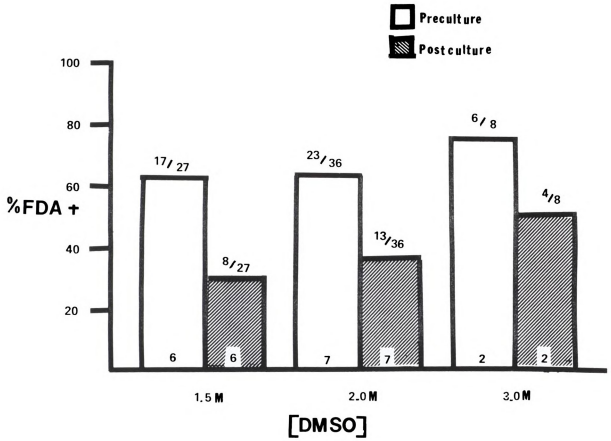

Figure 9. The effect of the concentration of DMSO on the viability of cryopreserved squirrel monkey ova. Viability was determined immediately after the ova were recovered and after the recovered ova were cultured for 3 hrs. The number in the data bars indicates the number of trials. The fractions above the data bars indicate the number of viable ova per recovered ova.

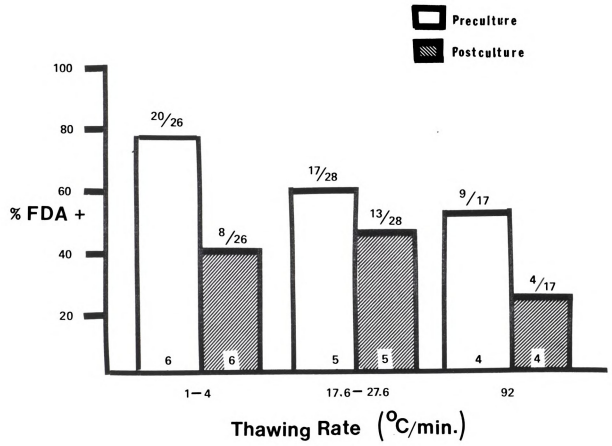

Figure 10. The effect of thawing rate on the viability of cryopreserved squirrel monkey ova. Viability was determined immediately after recovery from storage and after the ova were held in culture for 3 hrs. The number in the data bars indicates the number of trials. The fractions above the data bars indicate the number of viable ova per recovered ova.

TABLE 12

The Xenogenous Fertilization of Frozen Hamster and Squirrel Monkey Ova

Species	Treatment	Number of Rabbits	Ova Deposited	Ova Recovered[1]	Ova Fertilized[2]	Ova Cleaved[2]
Hamster	Control	8	273	143(52.4)	51(35.7)	11(7.6)
Hamster	Frozen	8	176	68(38.6)	8(11.8)	0
Squirrel Monkey	Control	10	94	34(36.2)	12(35.3)	0
Squirrel Monkey	Frozen	10	68	29(42.6)	8(27.6)	1(3.4)

[1]Figure in parentheses represents the percentage of ova recovered.

[2]Figure in parentheses represent a percentage of the recovered ova.

fertilized, by homologous sperm, in the rabbit oviduct. No cleavage of frozen hamster ova was observed after xenogenous fertilization. Squirrel monkey ova, when frozen under optimal conditions, showed no difference, when compared to nonfrozen controls, in their ability to be fertilized by homologous sperm in the rabbit oviduct ($\chi^2_1 = 0.1$).

The fertility of cryopreserved squirrel monkey ova was also tested by their ability to be fertilized in vitro. These results are shown in Table 13. No difference in maturation rate or fertilization rate was observed between control and frozen ova. This was determined by use of the Fishers Exact test.

TABLE 13

In Vitro Fertilization of Cryopreserved Squirrel Monkey Ova

Treatment	Number of Trials	Ova	Ova Matured	Ova Fertilized
Control	4	25	5	1 (20%)
Frozen	4	29	6	3 (50%)

DISCUSSION

Hamster ova are very elastic in their requirements for successful frozen storage. Quinn et al. (1982) showed that hamster ova survived freezing when they were slow cooled to -40°C, plunged into liquid nitrogen, and recovered by rapid thawing (≈500°C/min) or when they were slow cooled to -80°C, plunged into liquid nitrogen, and recovered by slow thawing (≈8°C/min). Viability was judged only by the appearance of normal morphology and the ability of zona pellucida free hamster ova to bind homologous or heterologous sperm. However, Quinn et al. did not show that other combinations of cooling and thawing rates would have any effect on the viability of frozen hamster ova.

In this present report, a high proportion of hamster ova survived slow (≈1-2°C/min) or rapid (≈92°C/min) thawing after being slowly cooled to -80°C and stored in liquid nitrogen. Therefore, both thawing rates allowed proper equilibration of solution between the ovum and extracellular fluid during thawing.

Slow cooling from seeding temperature to -80°C before storage in liquid nitrogen was not mandatory for survival of hamster ova. Hamster ova survived freezing equally well if they were slow cooled from -30°C to -40°C or if they were slow cooled from -10°C to -80°C before plunging into liquid nitrogen. From this data, it can be

inferred that the temperature range at which the necessary dehydration of hamster ova occurs, is between 30°C and 40°C.

Leibo et al. (1978) noted that the temperature at which intracellular ice formation occurs in mouse ova cooled rapidly in 1.0 M DMSO was -45°C. This formation of intracellular ice was correlated with ovum lethality. Furthermore, Rall et al. (1983) noted that the lethal event of rapid freezing occurred a few degrees (1-2°C) above the temperature at which intracellular ice was observed forming. The present data agree with these findings. Hamster ova must be slow cooled to at least -40°C or there will be insufficient dehydration in the ova and ice formation will occur at the nucleation temperature.

The choice of ≈92°C/min as the thawing rate to recover ova in the determination of the optimum slow cooling temperature range may have obscured the true temperature at which proper dehydration must occur. Rapid thawing prevents the regrowth of ice crystals that are lethal, upon the slow thawing of improperly dehydrated cells (Rall et al., 1980). Choosing 92°C/min buffered against the growth of ice crystals in cells in which insufficient dehydration had occurred. Furthermore, this thawing rate allowed sufficient time for fluid equilibration when cells, which had been cooled to -80°C, were thawed. Therefore, this thawing rate may allow more leeway in the requirements for hamster ova cryopreservation.

In contrast, squirrel monkey ova are not as flexible in their requirements for cryopreservation. Monkey ova slow cooled to -80°C in 1.5 M DMSO and stored at -196°C did not show the high survival rate observed with the hamster ova. Increasing the concentration of DMSO

from 1.5 M to 2.0 M increased the survival of monkey ova. The need for a higher concentration of DMSO for increased survival indicated that DMSO was improperly penetrating the ova and insuring proper protection. Increasing the temperature, at which the DMSO was added, from 0°C to 20°C allowed the proper penetration of DMSO into the monkey ova to insure proper protection. This need for the addition of DMSO at a temperature above 0°C has been observed for rat oocytes (Kasai et al., 1979), sheep embryos (Moore and Bolton, 1976), and cow embryos (Willadsen, 1976). Although reports show that mouse embryos also require DMSO to be added at temperatures higher than 0°C before freezing (Parkening et al., 1976; Wilmat, 1972), the addition of DMSO at 0°C before freezing is suitable for the storage of mouse embryos (Whittingham et al., 1972; Leibo et al., 1974; Whittingham, 1975).

The viability of hamster ova was determined by the ability of the ovum to exclude trypan blue. Trypan blue measures the integrity of the plasma membrane. However, it may overestimate viability because the plasma membrane is not the sole factor controlling viability of a cell (Tennat, 1964; Dolan, 1965). In this case, trypan blue did not prove to be an index of fertilizability of frozen hamster ova. Frozen-thawed hamster ova did not fertilize xenogenously, as well as the controls. Although previous reports have demonstrated that frozen-thawed zona pellucida free hamster ova were able to bind homologous sperm, as well as the controls (Flemming et al., 1979; Quinn et al., 1982), the process of fertilization requires the penetration of the zona pellucida by sperm. Damage to the zona pellucida during freezing and thawing is not detected by the vital dye, trypan blue.

Therefore, this undetected damage to the zona pellucida may account for the lowered fertility.

Frozen-thawed squirrel monkey ova, which were in vitro and xenogenously fertilized, gave results similar to those of the controls. The viability assay used to determine squirrel monkey ovum viability was FDA incorporation. This vital dye tests membrane integrity and the presence of esterases (Rotman and Papermaster, 1966). It has been used to test the viability of frozen-thawed HeLa S-C cells (McGrath et al., 1975), mouse embryos (Mohr and Trounson, 1980), and squirrel monkey ova and embryos (Chan et al., 1982).

The difference in fertility of frozen-thawed squirrel monkey and hamster ova may not be due to differences in the estimation of viability. FDA incorporation has the same limitations as trypan blue in that it only estimates two of the many factors necessary for cell survival (Dolan, 1965). Furthermore, FDA has not been observed to be a more conservative estimator of viability than trypan blue. In fact, FDA provides estimates of squirrel monkey ova viability similar to the estimates of hamster ova viability obtained with trypan blue (Hutz, 1983).

The differences in the fertility of frozen hamster and squirrel monkey ova could lie in the differences in the zona pellucida of the respective ova. The zona pellucida of the squirrel monkey ova could tolerate freezing better than the zona pellucida of hamster ova. This would not be detected by either of the vital dyes. Also, the differences could lie in the inability of the hamster ova to tolerate manipulation. Hamster embryos are very sensitive to manipulation in

culture. The slightest manipulation can place developing hamster embryo into unexplained refractoriness to development (Whittingham and Bavister, 1974). The additional manipulation involved in the freezing of the hamster ova may be the cause of refractoriness in development.

SUMMARY AND CONCLUSIONS

The aim of this present study was to develop a system for the cryopresrvation of hamster and squirrel monkey ova. This system would be used to create a readily accessible pool of ova for the production of embryos by in vitro and xenogenous fertilization. To achieve this end, hamster and squirrel monkey ova were placed in media containing various concentrations of DMSO. The ova were then cooled to various subzero temperatures before being stored in liquid nitrogen. Ova were recovered from cold storage by thawing at 1-4°C/min, 17.6-27.6°C/min, or 92°C/min. Viability of the thawed hamster and squirrel monkey ova was determined by the ovum's ability to exclude trypan blue and to incorporate FDA, respectively. After freezing by the optimum procedure, hamster ova and squirrel monkey ova were xenogenously fertilized by placing the ova into the oviducts of pseudopregnant rabbits with homologous sperm. Frozen squirrel monkey ova were also fertilized in vitro. The following conclusions can be made from this study:

1) Cryopreservation of hamster ova was achieved by using either PBS or TC-199 as the freezing medium.

2) The viability of frozen hamster ova was not affected by increasing the concentration of DMSO from 1.5 M to 2.0 M or if recovery of the frozen ova was accomplished by thawing at 1-2°C/min or 92°C/min.

3) The optimum temperature range for which hamster ova must be cooled slowly was very broad. The beginning of the slow cooling of hamster ova at -10°C, -20°C, or -30°C and the termination of the slow cooling at -40°C to -80°C did not alter the viability of frozen hamster ova.

4) Frozen hamster ova showed a decreased ability to be fertilized in the oviducts of pseudopregnant rabbits.

5) The temperature at which DMSO is added prior to cryopreservation is vital for successful freezing of squirrel monkey ova. Addition of DMSO at 20°C, in lieu of 0°C, significantly increased the viability of the squirrel monkey ova.

6) The viability of frozen squirrel monkey ova was not affected by increasing the concentration of DMSO from 1.5 M to 3.0 M added at 20°C.

7) Increasing the thawing rate of frozen squirrel monkey ova from 1-4°C/min to either 17.6-27.6°C/min or 92°C/min did not alter viability after freezing.

8) Frozen squirrel monkey ova showed no difference in their ability to be fertilized xenogenously or in vitro.

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APPENDIX A

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APPENDIX B

Vita

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DEGREES RECEIVED:	Bachelor of Science (1979) Cornell University Ithaca, New York Master of Science (1981) Michigan State University East Lansing, Michigan