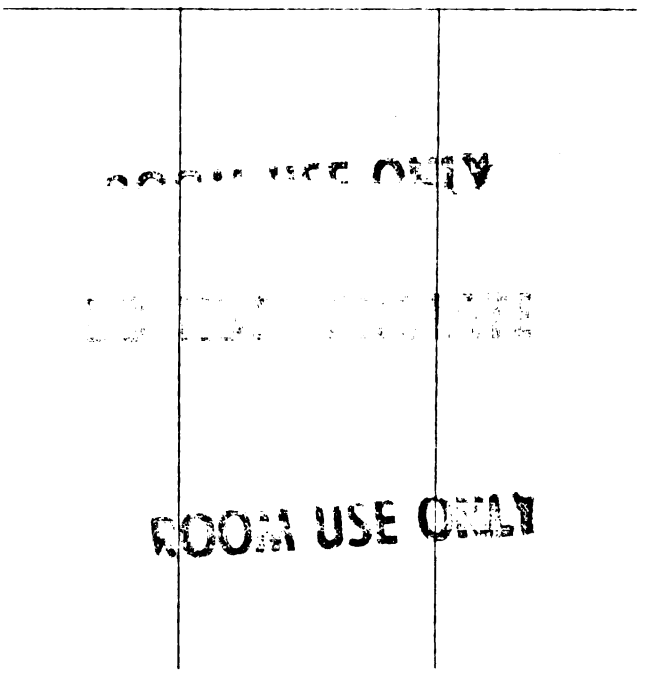

# EMBRYO PRODUCTION, FREEZING AND TRANSFER IN THE GOLDEN HAMSTER (MESOCRICETUS AURATUS)

By

Mundhir Taiyeb Ridha

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

1983

ABSTRACT

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The various developmental stages of hamster embryos (1-cell to morula) were studied as a model for embryo freezing and transfer. The survival rate of hamster embryos was examined by three different methods: 1) normal morphology, 2) trypan blue exclusion and 3) transfer techniques. The three tests were positively correlated. Viability of hamster embryos was significantly higher in tissue culture-199 (TC-199) than Dulbecco's phosphate buffere saline (PBS) medium. Optimum seeding temperature was above -9°C. Best results were obtained when hamster embryos were slowly cooled (0.33°C/min) and thawed (1.5°C/min) compared to rapid thawing (90°C/min). The developmental potential (to 6-day and 14-day old fetuses) of 1-cell and 2-cell embryos was significantly lower than other embryos. Superovulation resulted in lower implantation rates and a 9 h delay in ovulation in unfrozen embryos. With unfrozen embryos a higher implantation rate was observed in TC-199 than PBS and unilateral than bilateral transfer. Intrauterine transfer of 4- and 8-cell embryos was superior to intrabursal transfer. The implantation rates of unfrozen hamster embryos (2-cell to 8-cell) were similar in both horns. Migration of 1-cell embryos was significantly higher than 2-cell embryos. The transfer of 7 to 9 embryos per horn or oviduct resulted in a higher implantation

rate than 3 to 6 or 10 to 12 embryos per transfer. No significant difference was observed between the developmental stages of the embryo and implantation or pregnancy rates. Similar implantation rates were noted in young (14 wk) and mature (26 wk) recipient hamsters. Hamster embryos were found to be a good model for freezing and transfer experiments.

To Saeeda and Dukelow

with love and appreciation

ACKNOWLEDGMENTS

I wish to acknowledge those people who encouraged me in performing this work. Sincerest thanks are expressed to my great-hearted major advisor, Dr. W. Richard Dukelow, who gave me unlimited support, encouragement, and guidance during the entire course of this study.

Thanks and appreciation are expressed to my advisory committee: Dr. L.G. Clemens, Dr. Pericles Markakis, Dr. R.J. Aulerich and Mr. Richard Lehnert, for reading the thesis and providing valuable suggestions.

The author's appreciation is expressed to the Department of Animal Science and Faculty. Thanks are also extended to the graduate students of the Endocrine Research Unit and our secretary Ms. L.M. "Bonnie" Cleeves for their encouragement and assistance, and to Ms. Cathy Allen for her efforts in the preparation of this thesis.

Special thanks are extended to the greatest wife, Saeeda, and children, Ahmed and Ali, who showed faith, support and patience during my study.

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# INTRODUCTION

Embryo freezing and transfer techniques are useful tools for producing superior genetic stock in domestic animals. These techniques have been used to examine maternal-embryonic interaction and other aspects of implantation. The embryo itself is a special kind of organ; unlike most other cell types, it consists of a group of undifferentiated cells varying in size during early cleavage and having the potential to give rise to a complete new individual. Since the successful storage of mouse embryos in liquid nitrogen (Whittingham, et al., 1972; Wilmut, 1972) the techniques have been improved and applied to the storage of other embryos. Although the basic technique for embryo freezing is similar for all species, factors such as the rate of cooling and thawing, cell permeability, level and kind of cryoprotectant, seeding temperature, final storage temperature, cell size, stage of embryonic development and species sensitivity must be considered.

Unfrozen and frozen-thawed preimplantation embryos can be transferred to recipient females either surgically or nonsurgically. Advanced stages of embryos (morula and blastocyst) are normally transferred into the uterus, while the earlier stages of embryos are transferred to the oviduct. In rodents the early stages can be transferred to the ovarian bursa.

The ready availability of hamster embryos, in contrast to more expensive animals, make this species an attractive model in which to study the normal development of preimplantation embryos. The techniques used in this study for freezing preimplantation embryos were based on reports by Whittingham et al. (1972) and Tsunoda et al.

(1976). The goals of the present study were the following: 1) to develop a simple, repeatable and objective method for hamster embryo freezing and transfer; 2) to evaluate tests of frozen-thawed hamster embryos that correlate with their viability; 3) to define the optimal conditions for low temperature preservation and thawing of hamster embryos; and 4) to examine the effect of various factors on implantation of unfrozen embryos and these factors include: a) developmental stages of preimplantation embryos: b) recovery times of natural vs. superovulated embryos; c) superovulation vs. natural ovulation; d) intrabursal vs. intraoviductal transfer; e) unilateral vs. bilateral transfer; f) left horn vs. right horn; g) PBS vs. TC-199 medium; h) young (14 wk) vs. mature (28 wk) hamster; i) number of transferred embryos per transfer; j) embryo migration.

# REVIEW OF LITERATURE

## A. Embryo Freezing

### 1. Freezing Principles

In general, freezing causes death to most living organisms. Some biochemical reactions can be slowed or stopped by freezing, while others can be accelerated. Freezing is commonly used for preservation of cells and tissues. Normally, freezing leads to ice formation in the external medium and supercooling of the cell to -10°C or -15°C. This means that the membrane of the cell protects the supercooled interior of the cell against the growth of external ice. The intracellular vapor pressure is higher than the external ice because of the supercooled water that occurs. This high vapor pressure causes the cell to lose water in order to reach equilibrium with the external ice. The cell undergoes dehydration, concentrates the solutes and this leads to low intracellular aqueous vapor pressure. The events that occur during cooling primarily depend on the velocity of cooling and on the cell permeability (Mazur, 1970).

The critical cooling rate which produces internal ice depends on the ratio of the voume of the cell to its surface area and on its permeability to water. Large spherical cells are less permeable to water and should have a lower critical cooling rate than smaller more permeable cells. Exposure of the cell to temperatures below 0°C leads to supercooling. The cooling rate and cell permeability to water are two important factors which aid the cell in regaining equilibrium. Cells with a high permeability to water or

which are slowly cooled are equilibrated by the transfer of intracellular water to external ice, i.e., they equilibrate by dehydration. Conversely, cells with a low permeability to water or which are cooled rapidly equilibrate partly by intracellular freezing. Small crystals are produced which are enlarged during warming because of their high surface free energies. Freezing causes dehydration or intracellular crystal formation and both of these lead to loss of water and an increase in the intracellular and extracellular solute concentrations.

Bank and Maurer (1974) suggested that the high survival of frozen rabbit embryos was due to slow cooling, which permitted sufficient time for the embryos to equilibrate osmatically with the concentrated extracellular solutions. They found that the embryos were not sensitive to differences in the warming rates between 1.3°C and 25°C/minute and that survival decreased at warming rates above 25°C per minute. They noted that the thermal and the osmotic factors were important in determining rabbit embryo survival after freezing.

Maurer and Haseman (1976) indicated that the optimal conditions for freezing and thawing rabbit morulae were not the same as for 8-cell embryos. They also found that 2.8M or greater DMSO concentrations gave a low survival after thawing. This may be due to dehydration of the cells (Meryman, 1974) or to the toxic effect of high DMSO concentrations produced during freezing (Meryman, 1971). Whittingham (1977a) stated that the embryos of different species have different sensitivity to cooling depending on the stage of development. This was confirmed by Polge (1977) who found that cells were more sensitive to slow warming than to rapid warming when rapidly cooled. This was due to formation of ice crystals within the cells.

Leibo (1977) described the events that occurred during cooling and thawing. Lower temperatures result in ice crystal growth inside the cell leading to an increased concentration of dissolved solutes. Warming melts the ice and exposes the cell to solute dilution. Further, slow cooling to below the freezing point causes first, ice formation at a certain point below freezing and second, the quantity of ice nucleation increases to reach a chemical potential equilibrium for that subzero temperature. Third, the amount of ice formation increases in response to lowering the temperature of the solution causing increasingly concentrated solutes. These progressive changes continue as the temperature is lowered. Leibo (1977) stated that the osmotic responses of the mouse embryos are time and temperature dependent and all respond osmotically to maintain chemical potential equilibrium across their membranes. Leibo (1977) found that the total cell volume that an ovum contains when it freezes intracellularly is substantially less at lower than at high cooling rates. The occurrence of intracellular ice and the reduction in cell volume coincide with each other at the same ranges of cooling rates. Ova cooled at rates of 1°C/min or slower decrease in volume during cooling and few of them freeze intracellularly.

Mazur (1963) presented equations for the osmotic response of the cell to the increasing solute concentration produced by progressive freezing. The change in cell volume rate depends on the cell's surface area, the cell's original volumes the cell's permeability to water and finally the temperature coefficient of that permeability. By this model it is possible to measure intracellular ice formation during freezing. Leibo (1977) stated that this model

can approximately describe the actual response of mouse ova to freezing and also indicated that the response of ova and embryos to freezing is not unique. Whittingham (1977b) found that the reasons for the difference observed between species and developmental stages in the sensitivity of embryos to cooling to 0°C were unclear. Results from mouse studies have demonstrated that fast cooling rates produce ice formation intracellularly, which is incompatible with the survival of embryos when thawed slowly. Gradual lowering of temperature will allow enough time for the cell to gain osmotic equilibrium with the extracellular solution by progressive loss of water (Leibo and Mazur, 1978). In contrast, rapid cooling will not give sufficient time for the cell to remain in osmotic equilibrium, and, as the temperature is lowered, the cell contents will become increasingly supercooled. This causes the water to freeze intracellularly, which leads to death of the cell. Usually prior to freezing the embryo is mixed with dimethyl sulfoxide (DMSO) or glycerol. This protects the embryo against intracellular ice formation and gives a higher survival rate. The permeation of the solutes are temperature and concentration dependent. The greater the concentration and the higher the temperature of exposure, the faster the solute permeates the cell membrane. These cryoprotectants protect the cell from freezing damage by reducing the electrolyte concentrations by virtue of their colligative action (Mazur, 1979). They are usually present in molar concentration and, when the cells are cooled slowly with addition of one of the cryoprotectants, cell survival normally will be high.

2. Freezing of Preimplantation Embryos

a. Media

The primary requirement of the freezing medium is for the maintenance of embryonic viability during embryo collection, freezing, transfer and manipulation in vitro (Whittingham, 1979). The most suitable types of media are simple physiological salt solutions buffered with phosphate or Hepes and supplemented with glucose, pyruvate, bovine serum albumin or bovine serum and antibiotics (Whittingham, 1971b, 1979). These media normally have stable pH in air (7.2 - 7.4) and are superior to bicarbonate-buffered media previously used for most embryo transfers where the pH cannot be maintained in air alone (Whittingham, 1979). High pH and wide and rapid temperature fluctuations are detrimental to hamster embryo survival (Whittingham and Bavister, 1974). Phosphate and Hepes-buffered media are suitable for low temperature storage (-196°C) and also support the developmental stage of 8-cell embryos and morulae for at least two cleavage divisions at 37°C (Betteridge, 1977). Whittingham and Wales (1969) found that the addition of glycerol or DMSO to Ringer's medium at 4 to 5°C for 24 hours of storage had no effect on embryo survival. The return of embryos to normal size was more rapid with DMSO than with glycerol, perhaps due to the rapid movement of DMSO through the cell membrane causing a normal osmotic equilibration. All preimplantation stages of mouse embryos survived freezing and thawing best with Dulbecco's phosphate-buffered saline (PBS) supplemented with pyruvate, glucose, bovine serum albumin and antibiotics (Whittingham, et al., 1972; Wilmut, 1972; Whittingham, 1974). The same medium was used for 4- and 8-cell rabbit

embryos and morulae (Bank and Maurer, 1973; Whittingham and Adams, 1974), morulae and blastocysts of sheep (Willadsen et al. 1976), and cattle blastocysts (Wilmut and Rowson, 1973; Trounson et al., 1978).

Dulbecco's PBS supplemented with 0.1% glucose and 0.3% to 1.0% BSA or 10% heat-inactivated fetal calf serum was used as a freezing medium for zona-free hamster ova (Fleming et al., 1979). Ova were stored at -50°C for 3 and 120 days. Of the 152 frozen ova 84% showed normal morphological appearance upon thawing. Quinn et al. (1982) observed a significant difference in the penetrability of frozen-thawed hamster oocytes when comparing PBS and Hepes-buffered Tyrode's medium. Hamster oocytes frozen in Hepes-buffered Tyrode's medium survived much better (76%) when slow cooling was terminated at -80°C instead of -40°C (66%) before transfer to liquid nitrogen. In contrast, mouse embryos frozen in PBS showed no difference in survival whether slow cooling was terminated at -40°C or -80°C (Whittingham, et al., 1979). High penetration percentages observed in frozen hamster ova when Hepes-buffered medium containing 4 mg BSA/ml was used as a freezing medium (Quinn et al., 1982). Optimal survival rates of frozen hamster oocytes were obtained with PBS medium containing high protein (10% fetal calf serum)concentration (Fleming et al., 1979). The higher survival rates of hamster oocytes stored in HT6 freezing medium rather than PBS could be due to the difference in protein concentration and buffering system (Quinn, et al., 1982). Phosphate buffered medium is detrimental to survival of mouse embryos at 37°C (Quinn and Wales, 1973).

Whittingham et al. (1979) observed the highest survival levels of 8-cell mouse embryos (88%) after transfer to liquid nitrogen from -40°C, while with blastocysts 74% survival was obtained. The

highest survival rate (76%) was obtained when DMSO in PBS was diluted from the sample at 37°C and added either at 0°C or 37°C before freezing (Whittingham, 1974). Trounson et al. (1978) used PBS supplemented with 20% fetal calf serum and found that 52% of the frozen-thawed cow blastocysts continued normal development in vitro and 40% resulted in pregnancies when transferred to recipients. When PBS medium was supplemented with 50% rabbit serum, only 48% of the morulae appeared normal. Thirty-one morulae were transferred to six recipients and this resulted in higher pregnancy (100%) and implantation (39%) rates (Tsunoda et al., 1982). These observations indicate that there are other important factors besides the freezing medium that can affect directly embryo viability.

b. Seeding Temperature

Ice formation (seeding) in the freezing medium is usually induced either by touching the side of the freezing ampule with forceps precooled in liquid nitrogen or by adding crystals of frozen medium. The effect of seeding on embryo survival and the optimum temperature for seeding has not been explored extensively (Moore and Bilton, 1977). Usually when the embryo is either not seeded or seeded below the optimal seeding temperature, then the freezing medium will not freeze spontaneously. This results in supercooling and inadequate dehydration with an increased intracellular freezing (ice formation inside the cell) upon reaching the nucleation temperature. This is incompatible with the survival of the embryos, causing serious intracellular damage (Leibo, 1977; Leibo and Mazur, 1978). Whittingham (1978) observed that supercooling of 8-cell embryos

below -6°C markedly reduced embryo survival. Sheep morulae and blastocysts demonstrated high sensitivity to seeding temperature. No embryos developed in culture after seeding at -10°C or when not seeded while 30 to 50% of sheep embryos developed after seeding at -2.5, -5.0 or -7.5°C (Moore and Bilton, 1977). It is obvious that a certain amount of intracellular ice is compatible with the survival of mammalian embryos, since slowly cooled embryos when transferred directly from the freezing apparatus at -30°C and -40°C to liquid nitrogen survived only if rapidly thawed (Willadsen, 1977; Whittingham, 1978). These results clearly indicate the necessity for seeding at temperatures above -10°C.

Miyamoto and Ishibashi (1981a) studied the effect of seeding temperature on survival of frozen-thawed mouse morulae. They found that seeding at -4 to -8°C had no effect on the survival rate in the presence of 1.2 M ethylene glycol or 1 M glycerol. However, seeding at -10°C or below significantly reduced survival rates of frozen-thawed morulae. The developmental rate, in terms of live young, was markedly decreased when seeding was induced at -13°C than -4°C. These data indicate that the seeding temperature is critical for embryo survival and that seeding should be induced at a temperature slightly below the freezing point of the cryoprotectant.

c. Freezing and Thawing Rates

Above a critical cooling rate survival of cell decreases with increasing freezing rate (Mazur, 1970; Leibo and Mazur, 1971). This is a manifestation of cell injury due to intracellular ice formation as evidenced by electron microscopy (Walter et al., 1975; Leibo, 1977). Whittingham et. al. (1972) obtained high survival of mouse

preimplantation embryos (80%) after slow cooling (0.3 - 0.4°C/min) and slow warming (4 - 25°C/min). Very few embryos survived either after freezing at rates faster than 2°C/min or rapidly thawing the embryos after slow cooling at 0.3°C/min. When mammalian embryos are cooled rapidly they become more sensitive to slow thawing. This is due to the damaging effect of slow thawing, possibly by the growth of ice crystals within the embryos (Polge, 1977).

Leibo et al. (1974) showed that the survival of frozen 8-cell mouse embryos depends as much on warming rate as on cooling rate. Embryos cooled at 1.7°C/min survived when thawed at rates from 1 to 100°C/min, whereas embryos slowly cooled at 0.18°C/min or 0.2°C/min, required warming rate of 2°C/min to give 90% survival. If warmed at 200°C/min very few survived (Leibo, et al., 1974; Leibo, 1977). This means that in the frozen state embryos cooled at 0.2°C/min differ from embryos cooled at 2°C/min, indicating that during the freezing process changes occur in the embryos that only manifest themselves during thawing. Kasai et al. (1980) observed that when mouse morulae were cooled rapidly at 17°C/min and thawed rapidly at 360°C/min a higher percentage survived than if thawed slowly at 25°C/min. Following rapid freezing and thawing 70 to 82% of these embryos developed to fully expanded blastocysts. Willadsen et al. (1976) and Willadsen (1977) showed that a freezing rate of 0.3°/min and thawing rate of 10°C/min in medium containing 1.5M-DMSO resulted in 70% survival of late sheep morulae and early blastocysts. No embryos survived when the freezing rate was increased to 1°C/min. Different cooling and thawing rates were found to be necessary for 7- and 12-day cow blastocysts. None of the advanced blastocysts survived when the same

freezing and thawing rates of early blastocysts were applied to the freezing of 12-day old blastocysts. Higher survival rates of 12-day old blastocysts (advanced) in 2.5 M-DMSO medium were obtained when freezing rate increased to 1.2 - 2.4°C/min (Trounson, 1977).

The temperature at which intracellular ice occurs (nucleation temperature) depends on both the extent of cellular shrinkage and the composition of the solution within the cell. Freezing conditions in which cellular shrinkage is not complete allow intracellular ice to form and this correlates with cell death (Farrant, et al., 1977). The damage to the mammalian cells on freezing can be due to osmotic stress across cell membranes during or after thawing (Bank, 1973; Farrant, 1977). Damage to the embryos in the absence of cryoprotectant appears directly related to concentration of the electrolytes, whereas injury in the presence of the cryoprotectants seems related to volume changes (Mazur, 1977). Membranes at lower temperatures are less resistant to deformations produced by osmotic changes in the cell volume. The survival of mammalian embryos slowly frozen in a cryoprotectant can also depend on the thawing rate. Rapid thawing is more detrimental to embryo survival than slow thawing (Whittingham, et al., 1972; Wilmut, 1972; Leibo, et al., 1974). When 8-cell mouse embryos are cooled rapidly, 70% or more survived if they had first been cooled slowly to -50°C, but none survive if they are slowly cooled to only -30° (Leibo, et al., 1974). This is due to intracellular ice nucleation which occurs at -45°C (Leibo, et al., 1974; Leibo, 1977).

3. Fertility of Frozen Embryos

The temperature at which embryos are held *in vitro* is a very important factor in determining the viability of mammalian embryos. Alliston *et al.* (1965) reported an increase in post-implantation embryonic mortality after 1- and 2-cell rabbit embryos were maintained at 40°C for 6 h *in vitro*. Staples (1967) cultured day 5 rabbit blastocycst for 8, 16 and 24 h at 37°C and upon transfer to foster mother found that 40%, 28% and 0% developed respectively. Table 1 shows the viability of mammalian embryos after storage at temperatures between 0°C and 10°C. Chang (1947) demonstrated that rabbit embryos at the 2-cell stage were damaged by cooling to 5 or 0°C in less than 4 h. However, Mauer (1962) showed that 8-cell rabbit embryos and early morulae placed directly into medium at 0°C and cooled at 3°C/min or 0.02°C/min (stored for 2 days) gave viable young upon transfer. Mouse embryos seem to tolerate cold shock and show less susceptibility to changes in storage temperature (Whittingham and Wales, 1969).

Whittingham, *et al.* (1972) and Wilmut (1972) showed that frozen mouse embryos from 1-cell up to the blastocyst stage are capable of normal development upon thawing and transfer to foster mothers. Sixty-five percent of the recipients became pregnant and 40% of the embryos in the pregnant mice resulted in normal offspring. The 1-cell and 2-cell embryos were less sensitive to freezing and thawing than blastocysts. Similar results were obtained by Wilmut (1972) with frozen mouse blastocysts. The frozen blastocysts were tested by an *in vitro* culture technique and 80% survival was observed. Similar freezing regimens have been used for freezing rat, rabbit, sheep, goat and cow embryos (Whittingham, 1979). Table 2 shows the fertility of frozen

embryos. Whittingham and Bavister (1974) studied the development of fertilized hamster ova after in vitro culture and transfer to foster mothers. No viable young were obtained from these transfers. Experiments on freezing hamster oocytes showed that 87 to 94% of the frozen oocytes appeared normal after thawing. However, when these ova were fertilized in vitro, very high percentage showed polyspermy (Tsunoda, et al., 1976). Quin et al. (1982) found that the rate of decondensation of human spermatozoa was faster in freshly collected oocytes than in frozen-thawed ova. Parkening and Chang (1977) found that there was a significant difference in the fertilization rate of frozen-thawed ova from immature and mature mice, but this was not true in the rat. Rabbit embryos are more sensitive to osmotic changes during freezing and thawing. Dilution of the 1.6 M-DMSO at 37°C was necessary to obtain high survivals. Sixty-five percent of the frozen-thawed 8-cell embryos developed to blastocysts in culture, and subsequent transfer to suitable recipients resulted in 15% newborn (Bank and Maurer, 1973, 1974).

Cow and pig embryos show a high sensitivity to cooling temperatures above 0°C. Eight-cell cow embryos demonstrated the highest sensitivity to freezing (Wilmut et al., 1975). When cow blastocysts were thawed rapidly after slow cooling in 1.5 M-DMSO and following embryo transfer to recipients, two newborn calves were born (Wilmut and Rowson, 1973). Very low resistance was observed in pig embryos to cooling. When these were cooled to 0°C, none of them survived following thawing, and very few embryos survived below 15°C. Upon transfer to recipients, none resulted in pregnancy (Polge et al., 1974; Polge, 1977). The critical temperature which causes damage to pig embryos seems to be

TABLE 1

Viability of Cooled Embryos after Storage between 10° and 0° C Temperatures

| Species | Developmental Stage of Embryos | Medium | Temperature (°C) | Storage Period | Viable Young (Percent) | Reference |
|---|---|---|---|---|---|---|
| Mouse | 2-cell | PBS | 5 | 1 day | (23) | Whittingham and Wales (1969) |
| Mouse | 8-cell | KR + BSA | 10 | 1 day | (39) | Kiessling (1963) |
| Rabbit | 2-cell | Rabbit serum | 0 | 4 days | (6) | Chang (1948a) |
| Rabbit | 2-cell | Rabbit serum | 10 | 4 days | (15) | Chang (1948b) |
| Rabbit | Morulae | Rabbit serum | 10 | 4 days | (46) | Chang (1948c) |
| Rabbit | Morulae | Serum + saline + gelatin | 10 | 11 days | (28) | Hafez (1971) |
| Goat | 4-cell and early Morulae | BRS + egg yolk citrate | 10 | 22 h. | (37) | Otsuki et al. (1960) |
| Pig | 8-cell | PBS | < 15 | few h. | (0) | Wilmut (1972) |
| Cow | late Morulae | PBS | 0 | 4 h. | few | Wilmut et al. (1975) |

TABLE 2

Viability of Frozen Mammalian Embryos
at Subzero Temperatures

| Animal Species | Embryo Stage | Cryoprotective Agent and Concentration | Storage Temp. | Storage Time | Embryo Transferred Stage | Viable Young (Percent) | Reference |
|---|---|---|---|---|---|---|---|
| Mouse | 2-cell | 1.5 M-DMSO | -196 | 4 years | Blastocyst | (51) | Whittingham (1977a) |
| Mouse | 8-cell | 1.5 M-DMSO | -196 | 4 years | Blastocyst | (47) | Whittingham (1977a) |
| Mouse | 8-cell | 75% PVP | -79 | 30 min. | Blastocyst | (21) | Whittingham (1971b) |
| Mouse | 8-cell | 1.5 M-DMSO | -196 | 8 days | Blastocyst | (29) | Whittingham et al. (1972) |
| Mouse | Blast. | 1.5 M-DMSO | -196 | | Expanding Blastocyst | (50) | Whittingham (1977a) |
| Mouse | 8-cell & 4-cell | 1.5 M-DMSO | -196 | 31 hr. | Blastocyst | (5) | Schneider et al. (1974) |
| Rabbit | 8-cell | 2.0 M-DMSO | -196 | 18 hr. | Blastocyst | (26) | Maurer and Haseman (1976) |
| Rabbit | 8-cell | 1.6 M-DMSO | -196 | 3 hr. | Blastocyte | (15) | Bank and Maurer (1974) |
| Sheep | Morulae | 1.5 M-DMSO | -196 | 12 hr. - 1 month | Morulae & Blastocyst | (40) | Willadsen et al. (1976) |
| Sheep | Morulae | 1.5 M-DMSO 2.0 M-DMSO | -196 | 2-3 months | Morulae | (33) | Moore and Bilton (1976) |

around 15°C, since all embryos survive above this temperature and the damage at 15°C may be associated with lipid phase changes within the membranes (Polge, 1977).

B. Embryo Transfer

1. Natural and Superovulation for Embryo Production

Superovulation regimens have been used widely for induction of larger numbers of embryo from superior donors. This has important economic benefits especially in the livestock industry (Foote and Onuma, 1970; Betteridge, 1977; Saumande and Pelletier, 1975). Superovulation responses in animals are dependent on several factors including breed, nutrition, population sizes of ovarian follicles, seasonal variations and hormonal preparation used.

Preimplantation embryos are usually obtained from the female reproductive tract following natural or induced ovulation. In later case gonadotropins (PMS and LH or HCG) are used for maximum embryo recovery. Embryo collection is performed in small rodents by flushing the oviduct and uterus, whereas in primates, rabbits and large animals, laparotomy or laparoscopy can be used. Advanced embryonic stages in the cow and mare are recovered by non-surgical procedures but this is not possible in other farm animals due to the anatomical nature of the female reproductive tract (Betteridge, 1977; Brackett, 1978; Whittingham, 1979). The timing of ovulation by superovulation regimen varies according to the species (Whittingham, 1979). Several variables affect the recovery rates. These include: 1) dose and interval of gonadotropin, 2) route of injection, 3) stage of estrous cycle and 4) age of animal (Fleming and Yanagimachi, 1980; Mizoguchi and Dukelow, 1981).

Greenwald (1962) reported that injection of 30 iu PMS on day 1 of the estrous cycle of the hamster resulted in the ovulation of an average of 70 eggs. Golden hamsters superovulated by injection 5 to 25 iu PMS followed 44 to 48 h later with similar doses of HCG resulted in good recovery rates (Tsunoda, et al., 1976). Low recovery rates were observed (mean of 15 to 20 ova per hamster) in superovulated hamsters with a higher dose (40 iu) of PMS and HCG (Moore and Bedford, 1978). An average of 57 ova per hamster were recovered with 30 iu PMS followed with 30 iu of HCG 72-76 h later (Mizoguchi and Dukelow, 1981). Fleming and Yanagimachi (1980) found that the minimum interval between PMS and HCG to obtain 83% ovulation and an average of 36 ova per hamster ovulated was 44 h. Mizoguchi and Dukelow (1981) reported that superovulation significantly increased the number of degenerated oocytes in aged hamsters.

The developmental potential of embryos from superovulated and naturally ovulated animals is similar, since normal offspring were obtained from superovulated embryos. Experiments with hamsters (Fleming and Yanagimachi, 1980), mice (Smith and Chrisman, 1975) and rabbits (Maurer, et al., 1968), confirmed the normality of newborn animals resulting from superovulation. On the other hand, a higher incidence of chromosomal abnormality has been reported in superovulated preimplantation embryos in the mouse (Maudlin and Fraser, 1977), hamster (Mizoguchi and Dukelow, 1981) and rabbit (Fujimoto, et al., 1974). Takagi and Sasaki (1976) observed that superovulated embryos were unable to extrude the second polar body and they believed that the meiotic maturation of the oocyte might have been affected by superovulation. High polyspermic fertilization was observed in superovulated mouse

oocytes by Maudlin and Fraser (1977) and they suggested that the cortical reaction which prevents polyspermy is defective in superovulated eggs at least *in vitro*. High doses of PMS (30 iu) were found to induce unilateral pregnancies in hamsters. This effect is probably due to the alteration in the rate of gamete's or embryo's transport to the uterus (Fleming and Yanagimachi, 1980).

2. Site of Deposition and Type of Transfer

The transfer of preimplantation hamster embryos has been studied by several investigators (Blaha, 1964; Orsini and Psychoyos, 1965; Sato and Yanagimachi, 1972; Ghosh *et al*. 1982). Hamster embryos (from 1-cell to blastocyst stages) can be transferred to synchronous recipient females by three different routes: intrabursal (I.B.), intraoviductal (I.O.) and intrauterine (I.U.) transfer. The site of embryo deposition selected is dependent on the developmental stage of the embryo (Sato and Yanagimachi, 1972; Ghosh *et al*., 1982). The early preimplantation embryos from 1-cell to 8-cell stages are usually transferred I.O. to synchronous recipients (Whittingham, 1979). Tarkowski (1959) obtained successful I.O. transfer of all preimplantation stages when these embryos were transferred to recipient mice on the first day of pregnancy or pseudopregnancy. However, there is species variation in the ability of early preimplantation embryos to continue normal development in the recipient uterus. This usually depends on the developmental stage of the embryos and the uterine environment of the recipient (Whittingham, 1979). Noyes *et al*. (1963) found that the intrauterine transfer of 1- and 2-cell embryos in rats and mice resulted in degeneration of these embryos within 24 h following the

transfer, but Moore and Shelton (1964) reported that the early preimplantation embryos (from 2- to 8-cells) survived to term when deposited in the uterus of recipient sheep. Higher survival was obtained however, by the I.O. transfer of the same embryos. In mice and rats higher survival of transferred embryos is obtained with recipients that mated one day after the donors (Whittingham, 1979).

Moore and Bedford (1978) developed a new technique for transfer of hamster oocytes by depositing the ova in the ovarian bursa (intrabursal transfer) using a fine micropipette. They found that the majority of these ova passed into the oviduct within 15 min. A higher percentage of *in vivo* fertilization rate (96%) was observed with cumulus-oophorus covered ova transferred by this method. The majority of these eggs were found to be fertilized within 4 h when transferred at the time of ovulation. This technique was utilized for studying the effect of drugs on ovulation (Martin *et al.*, 1981). Orsini and Psychoyos (1965) reported 62% implantation when hamster blastocysts were transferred by I.U. route into progesterone-treated ovariectomized recipients. Sato and Yanagimachi (1972) were unable to get implantation of 1- and 2-cell hamster embryos transferred I.O. However, they obtained (80%) implantation in synchronized hamsters when donor blastocysts were transferred to the uterus. Of these, 70% developed normally. Higher survival rates were observed in mice when bilateral transfer was performed rather than unilateral transfer (Muller and Carter, 1973). Similar results were obtained in rabbits when frozen-thawed morulae were transferred bilaterally (48%) compared to unilateral transfer (23%) (Tsunoda *et al.*, 1982).

3. Transfer Media

The chemical composition, pH, storage period and the temperature of the transfer media are important variables that affect embryo survival (Maurer, 1976; Betteridge, 1977, 1981). Simple medium such as PBS with pH of 7.4 and supplemented with protein (usually 20% donor's serum), an energy source (glucose, pyruvate, lactate) and antibiotics maintain embryo viability in culture and support the subsequent embryonic development upon transfer (Betteridge, 1977). However, these media are unable to support all the developmental stages of the preimplantation embryos. Whittingham (1975b) was able to obtain complete preimplantation development in the mouse and also Maurer (1978) in rabbit when bicarbonate-buffered media was used as culture media.

Hamster and mouse blastocysts transferred in TC-199 and Whitten's media resulted in healthy newborn (Sato and Yanagimachi, 1972; Moler et al., 1979). Fifty percent implantation was reported in rabbits when PBS medium supplemented with 5% fetal calf serum was used as the transfer medium for rabbit blastocysts (Rottmann and Lampeter, 1981). Moler et al. (1979) used Whitten's medium at 37°C for non-surgical embryo transfer in mice. The transfer of 278 blastocysts to 30 recipients resulted in a 90% pregnancy rate and 45% live young. Table 3 shows the effect of transfer media on survival of unfrozen mammalian species at temperatures between 25°C and 37°C.

The early preimplantation embryos of hamster, rat and guinea pig showed higher sensitivity to culture media and in vitro manipulation. This means they cannot tolerate in vitro culture compared to late stages of embryonic developement (Sato and Yanagimachi, 1972; Whittingham and Bavister, 1974; Whittingham, 1978).

TABLE 3

Viability of Mammalian Preimplantation Embryos
in Various Media at Temperature between 25°C and 37°C

| Animals | Developmental Stage of Embryos | Transfer Medium | Percent Viable Young | References |
|---|---|---|---|---|
| Mice | 2-cell | Chemically defined medium | 31 | Whitten & Biggers (1968) |
| Mice | Morulae | Synthetic medium | 37 | Hoppe & Pitts (1973) |
| Mice | Blastocysts | Whitten's medium | 45 | Moler et al. (1979) |
| Hamster | Blastocysts | TC-199 | 56 | Sato & Yanagamachi (1972) |
| Rat | 8-cell | Brinster's medium | 24 | Maurer (1976) |
| Rabbit | 4-cell | Bovine and rabbit serum | 50 | Maurer et al. (1970) |
| Rabbit | Blastocysts | Modified F10 with serum | 40 | Staples (1967) |
| Sheep | Morulae | Sheep serum or TC-199 | 16 | Moor & Cragle (1971) |

4. Age of Recipient

The degree of synchronization between donor and recipient is one of the most important factors for successful embryo transfer (Sato and Yanagimachi, 1972; Adams, 1973; Fukumitsu and Sugie, 1974; Betteridge, 1977; Whittingham, 1979). In small rodents implantation takes place within a short period of time after the entrance of the embryo to the uterus, therefore successful transfer depends on synchronous embryo transfer. Asynchronous transfer in rodents is successful only when the recipient females are mated one day later than the donors. This type of synchronization is useful for embryos that show developmental delay during handling in vitro (Whittingham, 1979). Sato and Yanagimachi (1972) demonstrated that older hamster embryos (4-day blastocysts) survived better after embryo transfer than younger embryos (3-day embryos, 4- or 8-cell embryos). The transplantation of 8-cell embryos from aged donor hamsters to young recipients resulted in a 49% survival rate. However, only 8% of embryos of the same stage developed into fetuses when transferred to older recipients (Blaha, 1964). Although the transfer of hamster blastocysts to ovariectomized progesterone-treated (artificially delayed implantation) recipients resulted in live fetuses, lower rates of fetal development were observed when the reproductive stages of the donor and recipient animals were asynchronous (Orsini and Psychoyos, 1965). A high pregnancy rate (100%) was observed in synchronized recipient hamsters receiving blastocysts compared to 4- and 8-cell embryos (50% and 87% respectively, (Sato and Yanagimachi, 1972). Ghosh et al. (1982) found no significant difference in implantation rates of 2-cell and 8-cell embryos in mature golden hamster (61% vs 64% respectively).

A low implantation rate was reported with 8-cell mouse embryos from donors 2.5 days postcoitus, transferred to recipient 3.5 days postcoitus, and the removal of the zona pellucida did not increase the implantation rate (McLaren, 1969). Toyada and Chang (1974) reported that of 203 2-cell embryos fertilized *in vitro* and transferred to oviducts of 2-day pseudopregnant rats, only 21% showed fetal development. Adams (1973) studied the effect of asynchronization between donors and recipients on rabbit embryo survival. He showed that the age of the transferred embryos was important for the maintenance of the corpus lutea, which became critical around 14 days postcoitus. Embryos up to 3 1/2 days younger than the corpus lutea were luteotrophically competent whereas those 5 1/2 days younger were not. Whittingham and Adams (1976) attributed the low survival of frozen-thawed rabbit embryos after transfer to damage to the zona pellucida during freezing or unfavorable synchronization between embryos and recipients. In general the fertility of preimplantation embryos decreases with the increasing age of donors and recipients. Many workers observed a marked decrease in embryonic development, implantation rate, litter size and fecundity in older hamsters (Soderwall *et al.*, 1960; Connors, 1969, 1972; Parkening and Soderwall, 1973).

5. Number of Embryos Transferred

Sato and Yanagimachi (1972) transferred 3 to 5 hamster embryos (4-, 8-cell and blastocysts) to each uterine horn. Of 206 embryos 63 percent implanted and of these 73 percent developed into fetuses. The transfer of 78 rat embryos (7 to 8 embryos per transfer) I.O. resulted in 42% implantation, 27% fetuses and 25% viable young

(Toyoda and Chang, 1974). The preimplantation and postimplantation losses of the embryos in this study were 57% and 15% respectively. They reported that rat ova fertilized *in vitro* are capable of normal development when transferred at the 2-cell stage. Table 4 shows the development of mammalian embryos and the effect of the number of embryos transferred on development. Maurer *et al.* (1970) found that rabbit embryos transferred (2 to 5 per oviduct) after 62 h in culture gave 80 percent pregnancy rate compared to 89% pregnancy rate with direct transfer. The period of pseudopregnancy in the recipient rabbit is an important factor that affects the transferred embryos. When 5 to 10 morulae were transferred to each uterine horn in a 3 to 7 day pseudopregnant rabbit, it resulted in 78% pregnancy and 39% newborn. Only 18.7% became pregnant and 1.7% gave newborn when embryos were transferred to 9-11 days pseudopregnant rabbits (Adam, 1971).

Studies in pigs showed that the transfer of large number of embryos to recipient oviducts is not a major factor that limits litter size. Recipients receiving 24 embryos per oviduct have higher pregnancy rate compared to recipients receiving 12 embryos at 25 days of gestation. The 24-embryo recipient gilts were found to have from 14 to 23 normal embryos per litter at slaughter (Pope *et al.*, 1972).

6. Embryo Migration

There are no reports in the literature on this subject in rodents. However, embryo migration is well documented in the pig (Anderson and Parker, 1976; Pope *et al.*, 1982 a, b), ewe (Scanlon, 1972) and cow (Rowson *et al.*, 1971; Scanlon, 1972). Pope *et al.* (1982a) studied the relationship between myometrial contractility and intrauterine

TABLE 4

Normal Development of Unfrozen Mammalian Embryos Following Embryo Transfer

| Species | Developmental Stage | No. Transf. per Horn or Oviduct | Total No. Transf. | Implantation | No. Fetuses | Viable Young | References |
|---|---|---|---|---|---|---|---|
| Mice | Blastocyst | 5 (I.U) | 450 | 225 (50%) | | | Humphrey (1967) |
| Mice | Blastocyst | 6 (I.U) | 322 | | 176 (54%) | | Fiser and Macpherson (1982) |
| Rat | 2-cell | 7-8 (I.O) | 78 | 33 (42%) | 21 (27%) | 20 (25%) | Toyoda and Chang (1974) |
| Hamster | 4-cell | 3-5 (I.U) | 64 | 23 (36%) | 19 (30%) | | Sato and Yanagimachi (1972) |
| Hamster | 8-cell | 3-5 (I.U) | 62 | 41 (66%) | 30 (48%) | | |
| Hamster | Blastocyst | 3-5 (I.U) | 80 | 66 (82%) | 46 (57%) | | |
| Hamster | Blastocyst | 3-5 (I.U) | 16 | | | 9 (56%) | |
| Rabbit | Morulae | 2-5 (I.O) | 100 | 17/19 (89%) pregnancy | | 54 (54%) | Maurer et al. (1970) |
| Rabbit | Morulae | 5-10 (I.U) | 175 | 18/23 (78%) | | 69 (39%) | Adam (1971) |
| Sheep | Morulae | one (I.U) | 9 | 7 (78%) | 7 (78%) | 5 (55%) | Peters et al. (1977) |
| Sheep | 2-, 4-, 8-cell | two (I.O) | 324 | 166 (51%) | | 117 (36%) | Moore and Shelton (1964) |
| Sheep | 1-, 2-, 4-cell | 1-2 (I.O) | 40 | 28 (70%) | 24 (60%) | | Wilmut and Sales (1981) |
| Sheep | Blastocyst | 1 (I.U) | 15 | | | 10 (66%) | |
| Pig | 1-, 2-, 4-cell | 12 (I.O) | 166 | pregnancy rate (71.4%) | (56.7%) | 94 (56%) | Pope et al. (1972) |
| Cow | Morulae and Blastocyst | 2 per one horn (I.U) | 22 | 11/15 (73%) pregnancy rate | | (45%) twin | Rowson et al. (1971) |
| Cow | Morulae and Blastocyst | 1 per each horn (Bilateral) | 22 | 13/18 (72%) | | (73%) twin | |

embryo migration in pigs. They found that myometrial contractility increased with embryo migration. In vitro studies showed that day 12 pregnant horn flushings overcame significantly the inhibitory effects of indomethacin on myometrial contraction. They found a short-acting substance in the pregnant horn flushings that mimicked the stimulatory effect of embryo on myometrial contraction. It was concluded that the factor responsible for uterine contraction was hormonal in nature (Pope et al., 1982a). The popular hypothesis for the mechanism of embryo migration is the peristaltic contraction of the myometrium that facilitates migration of the embryos (Boving, 1971; Pusey et al., 1980; Pope et al., 1982b). It was demonstrated that estradiol-17B and histamine are involved in intrauterine migration of porcine embryo. When silastic beads were impregnated with cholesterol or estradiol ($E_2$) after 5 days in the uterus, the beads which were impregnated with $E_2$ migrated significantly farther than those impregnated with cholesterol (Pope et al., 1982b).

## MATERIALS AND METHODS

### A. Animals

Healthy mature cycling golden hamsters (Mesocricetus auratus) three to six months of age (Charles River Laboratories, Wilmington, Mass.) were housed under a controlled 14 h:10 h (light:dark) cycle (light 0600 to 2000). Animals showing at least three consecutive 4-day estrus cycles were used.

### B. Embryo Recovery and Handling

Donor females were housed with fertile males for 12 h from 1900. Mating was confirmed by lordosis response and vaginal aspiration and examination for sperm the following morning. The donors were killed by cervical dislocation 26 to 31 h later (to recover 1-cell embryos), 35 to 43 h (to recover 2-cell embryos), 52 to 69 h (to recover 4-cell embryos), 68 to 71 h (to recover 8-cell embryos) or 77 h (to recover morulae) after mating. Embryos were flushed from the oviducts and uterine horns with a 30 ga. needle using either TC-199 medium (GIBCO, Grand Island, NE) or Dulbecco's Phosphate Buffered Saline (PBS, GIBCO Laboratories, Grand Island, NE). Hamster preimplantation embryos from 1-cell to the morula-stages were recovered as shown in figure 1 either from the oviduct or uterine horn.

### C. Freezing Techniques

The preimplantation embryos were washed twice with fresh medium following recovery. They were placed in an ampule containing 0.1 ml of the freezing medium (TC-199 or PBS) at 0°C. Next, 0.1 ml of

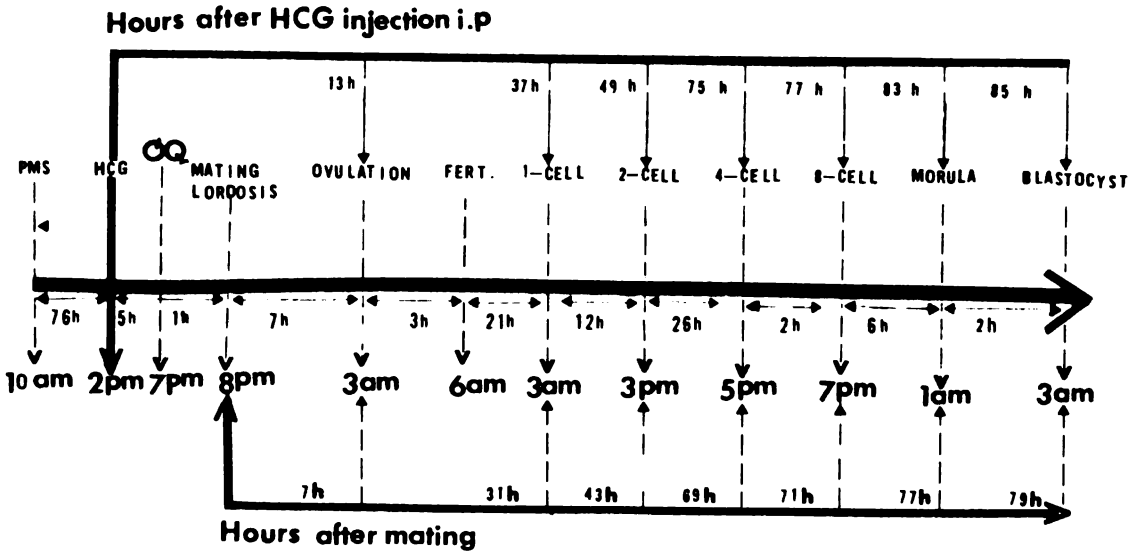


Figure 1. Embryo recovery times from golden hamster

3 M dimethylsulphoxide (DMSO) was added at 0°C. The final concentration of DMSO in the ampule was 1.5M. The ampule was sealed and placed in a 95% ethanol bath at 0°C and then seeded from -5°C to -10°C. Seeding was induced by immersing a forceps in liquid nitrogen and touching the ampule. The seeded embryos were cooled at a rate of 0.33°C/min (from 0.30 - 0.36°C/min) and when the final temperature of the ampule reached -85°C (from -80° to -90°C) the ampule was plunged into liquid nitrogen at -196°C and stored from 1 to 90 days.

The freezing system which was used in this study consisted of an unsilvered Dewar flask of six Torr vaccuum. Three hundred ml of 95% ethanol was placed in the Dewar flask. The Dewar flask was then placed inside a larger silvered flask which contained liquid nitrogen. The depth of the Dewar flask was controlled by a laboratory elevator jack. The temperature of the seeding ampule was recorded at 15 minute intervals. The freezing rate was calculated as a function of temperature and time.

Slow thawing was performed by placing the frozen ampule in a 600 ml pyrex beaker containing 300 ml of 95% cold ethanol at -125°C. The ampule was allowed to thaw slowly at a rate of 1.5°C/min. Rapid thawing was performed by direct transfer of the frozen ampule from the liquid nitrogen into an ice-bath (0°C). This gave a thawing rate of 90°C/min. As soon as the ampule thawed the DMSO was diluted by stepwise addition of 0.2, 0.2 and 0.4 ml of the medium at 30 second intervals and finally 1 ml of the medium. The ampule was washed again with 1 ml of medium. The washed medium was placed in a watch glass and examined for embryo recovery under a dissecting microscope. The thawed embryos were examined for morphological normality and trypan

blue exclusion (0.2%TpB in PBS). The tested embryos were washed twice with fresh medium and prepared for embryo transfer.

D. Embryo Transfer

1. Basic Technique for Transfer

Recipient hamsters were anesthetized with 0.12 ml pentabarbitol-sodium (60 mg/ml) intraperitoneally. Embryos were transferred to the ovarian bursa (I.B.), oviduct (I.O.) or uterus (I.U.) of the recipients by making a dorsolateral incision through the abdominal wall. The embryos from 1- to 8-cell were transferred through fine micropipette into ovarian bursa or oviduct. The 4-cell, 8-cell and the morulae were injected into the uterine horn through a micropipette or a 26 gauge needle connected to PE-20 intramedic polyethylene tubing (Clay Adams, Becton, Dickinson and Company, Parsippany, NJ). The other end of polyethylene tube was joined to a one ml tuberculin syringe or mouth piece. The embryos were also transferred to the uterine horn through a fine micropipette connected to a plastic mouth tube. The micropipette and the polyethylene tube were checked after each embryo deposition to assure that all embryos were transferred. The recipients were killed on day six or fourteen following the transfer and examined for implantation sites and embryonic development.

2. Superovulation and Recipient Synchronization

The day of postovulatory vaginal discharge was designated as day 1 of the cycle. Females were superovulated with an intraperitoneal injection of PMS (Sertropin, Teizo Company, Ltd., Tokyo, Japan). Twenty-five IU/100 gm of body weight at 1000 on any day of the

4-day cycle, followed 76 h later by an intraperitoneal injection of HCG (APL, Ayerst Laboratories, Montreal, Canada), 25 IU/100 gm of body weight at 1400 . Mating was as described earlier. The period of synchronization was the same for the donor and recipient groups. Recipients were ear marked, shaved, anesthetized and prepared for embryo transfer.

3. Age of Animals

The donor and recipient groups were both superovulated and synchronized. According to the age of donor and recipient, the animals were divided into two groups, namely young 14 wk old (12 to 16 wk) and mature 26 wk old (24 to 28 wk). The effect of age of animals on implantation rates was studied.

4. Number of Embryos Transferred

The effect of the number of embryos per each transfer on the implantation rate and embryo viability was examined. Recipients were divided into three subgroups depending on the number of transferred embryos, and each subgroup received 3-6, 7-9 and 10-12 embryos per transfer respectively.

Chi-square, Bonferroni chi-square and T-test were used for statistical analysis of the data (Gill, 1978). If the level of significance was not stated otherwise in the table the $p>.05$ was used.

5. Embryo Migration

To study the migration of early preimplantation embryos, all the surgical transfers were made unilaterally either to left or right ovarian bursa, oviduct or uterine horn. The recipients were killed on

day 6 or 14 of pregnancy and implantation sites were checked in both horns of the uterus. Migration of embryos to either left or right horn was counted and the interaction between the developmental stages and migratory capability of the embryos was also noticed.

# RESULTS

## 1. Media

The viability of various preimplantation embryos was not affected by freezing medium TC-199 (Table 5). The viability of 1-cell and 2-cell compared to morulae were significantly affected by PBS ($p<.01$) when used as a freezing medium (Table 6). The developmental stages of the embryos showed different responses ($p<.001$) to PBS medium (Table 6). The fertility of frozen-thawed embryos was determined by the embryo transfer technique. The fertility of these embryos was not affected by the medium (Tables 7 and 8). The survival of hamster embryos in TC-199 in terms of normal and $TpB^-$ embryos was significantly higher ($p<.01$ and $p<.001$ respectively) than in PBS medium (Table 9).

## 2. Seeding Temperature

Hamster preimplantation embryos were seeded at a temperature range from -5°C to -23°C. The effect of seeding temperatures on embryo survival was studied (Figures 2,3,4, 5 and 6). The optimal seeding temperature for 1-cell, 2-cell, 4-cell, 8-cell and morula was shown to be -5, -7, -9, -9 and -5 respectively. The survival of preimplantation embryos in terms of embryo normality, $TpB^-$ and implantation rate was lowered when the seeding temperature was below -10°C. Seeding temperatures between -5°C and -10°C did not have a deleterious effect on hamster preimplantation embryos (Figures 2 to 6). The numbers of normal, $TpB^-$ and implanted embryos were significantly ($p<.01$) increased when the embryos were seeded at temperatures above 10°C.

TABLE 5

*Viability of Frozen-Thawed Hamster Preimplantation Embryos in TC-199

| Embryo Stage | Number Frozen | Number Thawed (%) | Number Normal (%) | Number $TpB^-$ (%) | Number Donors |
|---|---|---|---|---|---|
| 1-cell | 149 | 114 (77) | 100 (88) | 106 (93) | 10 |
| 2-cell | 102 | 88 (86) | 72 (82) | 81 (92) | 8 |
| 4-cell | 42 | 33 (79) | 27 (82) | 28 (85) | 4 |
| 8-cell | 105 | 90 (86) | 76 (84) | 77 (86) | 9 |
| Morula | 50 | 35 (70) | 28 (80) | 28 (80) | 4 |
| Total | 448 | 360 (80) | 303 (84) | 320 (89) | 35 |

* $p>.05$

TABLE 6

Viability of Frozen-Thawed Hamster Preimplantation Embryos in Dulbecco's Phosphate Buffer Saline (PBS)

| Embryo Stage | Number Frozen | Number Thawed (%) | Number Normal (%) | Number $TpB^-$ (%) | Number Donors |
|---|---|---|---|---|---|
| 1-cell | 357 | 264 (74) | 192[a] (73) | 194 (73) | 24 |
| 2-cell | 94 | 68 (72) | 46[a] (68) | 52 (76) | 7 |
| 4-cell | 43 | 37 (86) | 32 (86) | 32 (86) | 5 |
| 8-cell | 91 | 53 (58) | 41 (77) | 44 (83) | 8 |
| Morula | 164 | 133 (81) | 116 (87) | 102 (77) | 13 |
| Total | 749 | 555 (74) | 427[b] (77) | 424 (76) | 57 |

a = $p<.01$ Significantly different from morula

b = $p<.001$ Morphologically normal embryos vs. developmental stages

TABLE 7

*Fertility of Frozen-Thawed Hamster Embryos in TC-199

| Embryo Stage | Number Transferred | Number Implanted (%) | Number Recipient | Number Pregnant (%) |
|---|---|---|---|---|
| 1-cell | 34 | 25 (74) | 5 | 4 (80) |
| 2-cell | 36 | 28 (78) | 4 | 4 (100) |
| 4-cell | 20 | 16 (80) | 3 | 3 (100) |
| 8-cell | 42 | 35 (83) | 8 | 8 (100) |
| Morula | 17 | 14 (82) | 3 | 3 (100) |
| Total | 149 | 118 (79) | 23 | 22 (96) |

* $p > .05$

TABLE 8

*Fertility of Frozen-Thawed Hamster Embryos in PBS

| Embryo Stage | Number Transferred | Number Implanted (%) | Number Recipient | Number Pregnant (%) |
|---|---|---|---|---|
| 1-cell | 88 | 66 (75) | 15 | 13 (86) |
| 2-cell | 25 | 20 (80) | 5 | 5 (100) |
| 4-cell | 10 | 8 (80) | 2 | 2 (100) |
| 8-cell | 24 | 21 (87) | 4 | 4 (100) |
| Morula | 48 | 35 (73) | 9 | 8 (88) |
| Total | 195 | 150 (77) | 35 | 32 (91) |

* $p>.05$

TABLE 9

Fertility of Frozen-Thawed Hamster Embryos in Two Freezing Media
(PBS vs. TC-199)

| Freezing Medium | Number Frozen | Number Thawed | Number Normal | Number $TpB^-$ (%) | Number Transferred | Number Implanted | Number Recipient | Number Pregnant |
|---|---|---|---|---|---|---|---|---|
| PBS | 749 | 555 (74) | 427 (77) | 424 (76) | 195 | 150 (77) | 35 | 32 (91) |
| TC-199 | 448 | 360 (80) | 303 (84)[a] | 320 (89)[b] | 149 | 118 (79) | 23 | 22 (96) |
| Total | 1197 | 915 (76) | 730 (80) | 744 (81) | 344 | 268 (78) | 58 | 54 (93) |

a = $p<.01$ Significantly higher than in PBS

b = $p<.001$ Significantly higher than in PBS

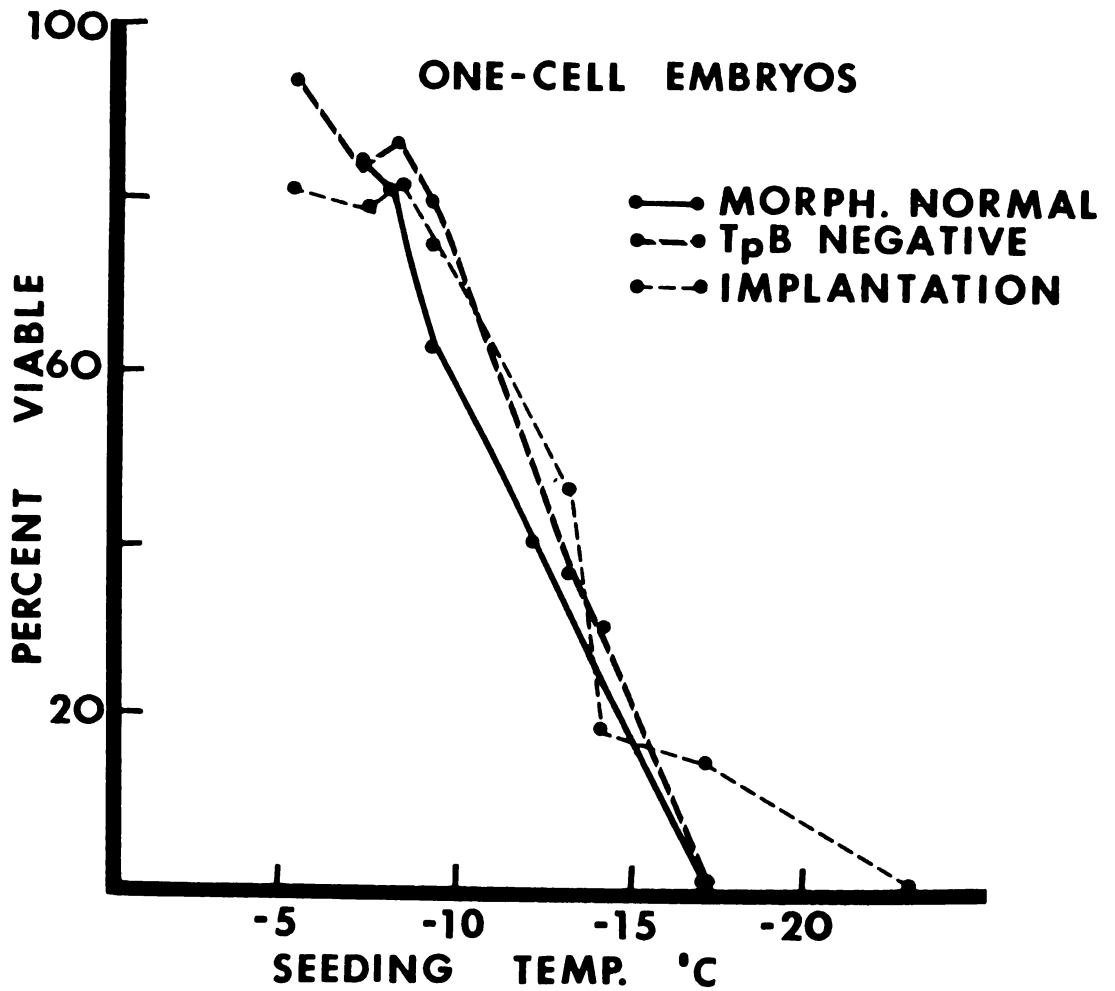


Figure 2. Survival of 1-cell hamster embryos seeded at temperatures between -5°C and -23°C. Each point represents 2 - 3 replicates. $p < .001$: seeding temperature -5 to -10°C vs. -11 to -15°C; $p < .005$: implantation significantly different from morphological test and trypan blue reaction at seeding temperature -11 to -15°C.

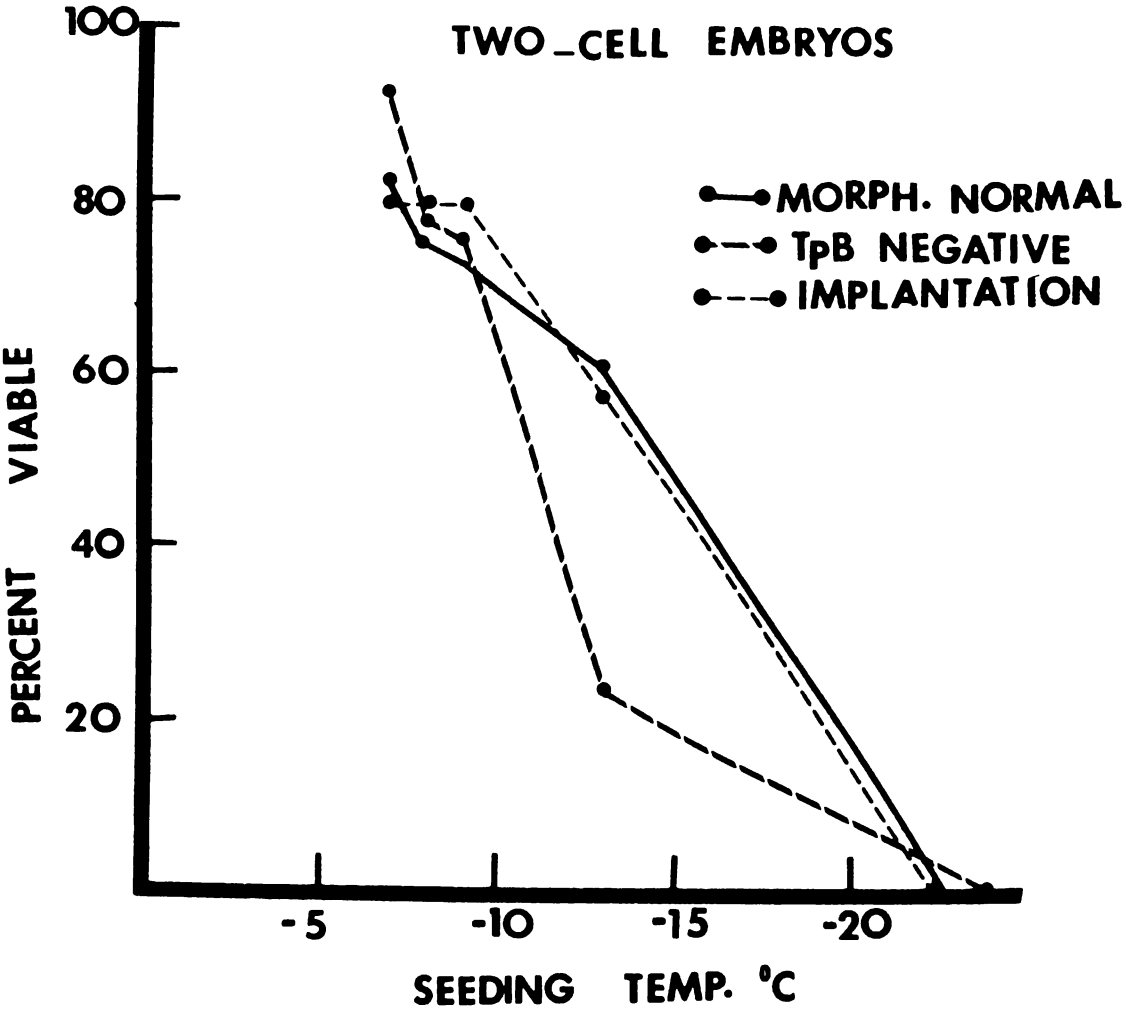


Figure 3. Survival of 2-cell hamster embryos seeded at temperatures between -7 and -23°C. Each point represents 2-3 replicates. $p < .001$: seeding temperature -7 to -10°C vs. -11 to -15°C; implantation significantly different ($p < .005$) from morphological test and trypan blue reaction at seeding temperature -11 to -15°C.

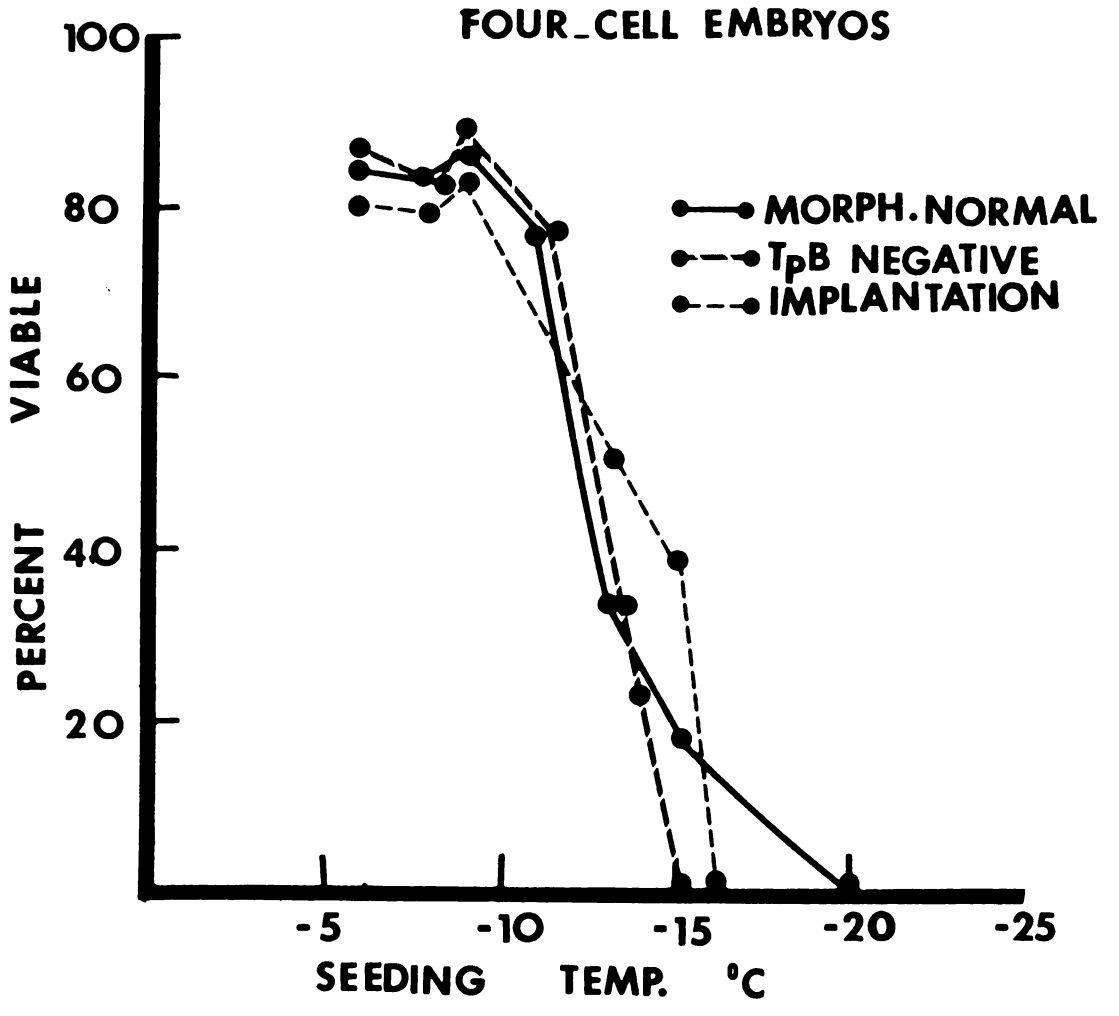


Figure 4. Survival of 4-cell hamster embryos seeded at temperatures between -6°C and -20°C. Each point represents 2-3 replicates. $p < .001$: seeding temperature -6 to -10°C vs. -11 to -15°C; implantation signficantly different ($p < .005$) from morphological test and trypan blue reaction at seeding temperature -11 to -15°C.

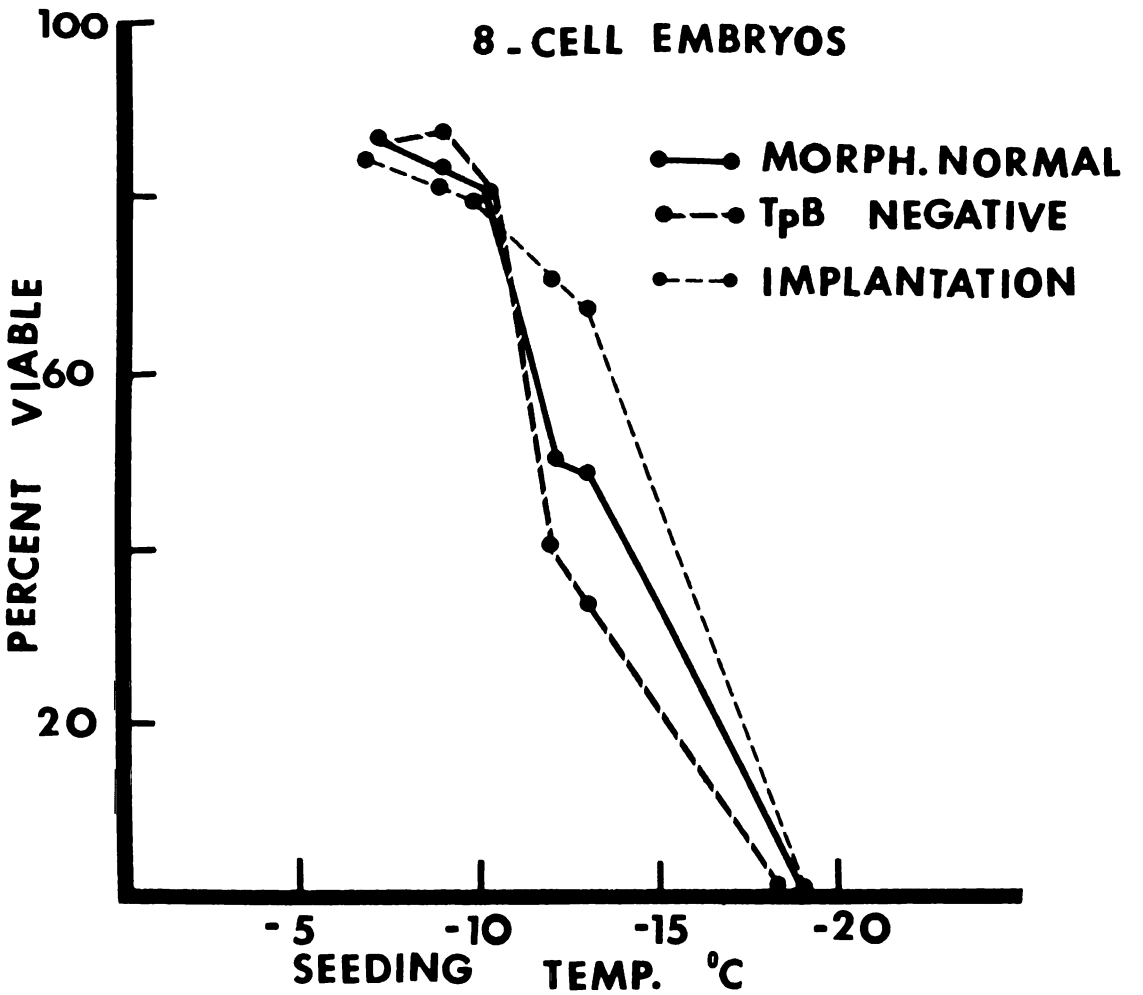


Figure 5. Survival of 8-cell hamster embryos seeded at temperatures between -7°C and -19°C. Each point represents 2-3 replicates. $p < .001$: seeding temperature -7°C to -10°C vs. -11°C to -15°C; implantation significantly different ($p < .005$) from morphological test and trypan blue reaction at seeding temperatures -11 to -15°C.

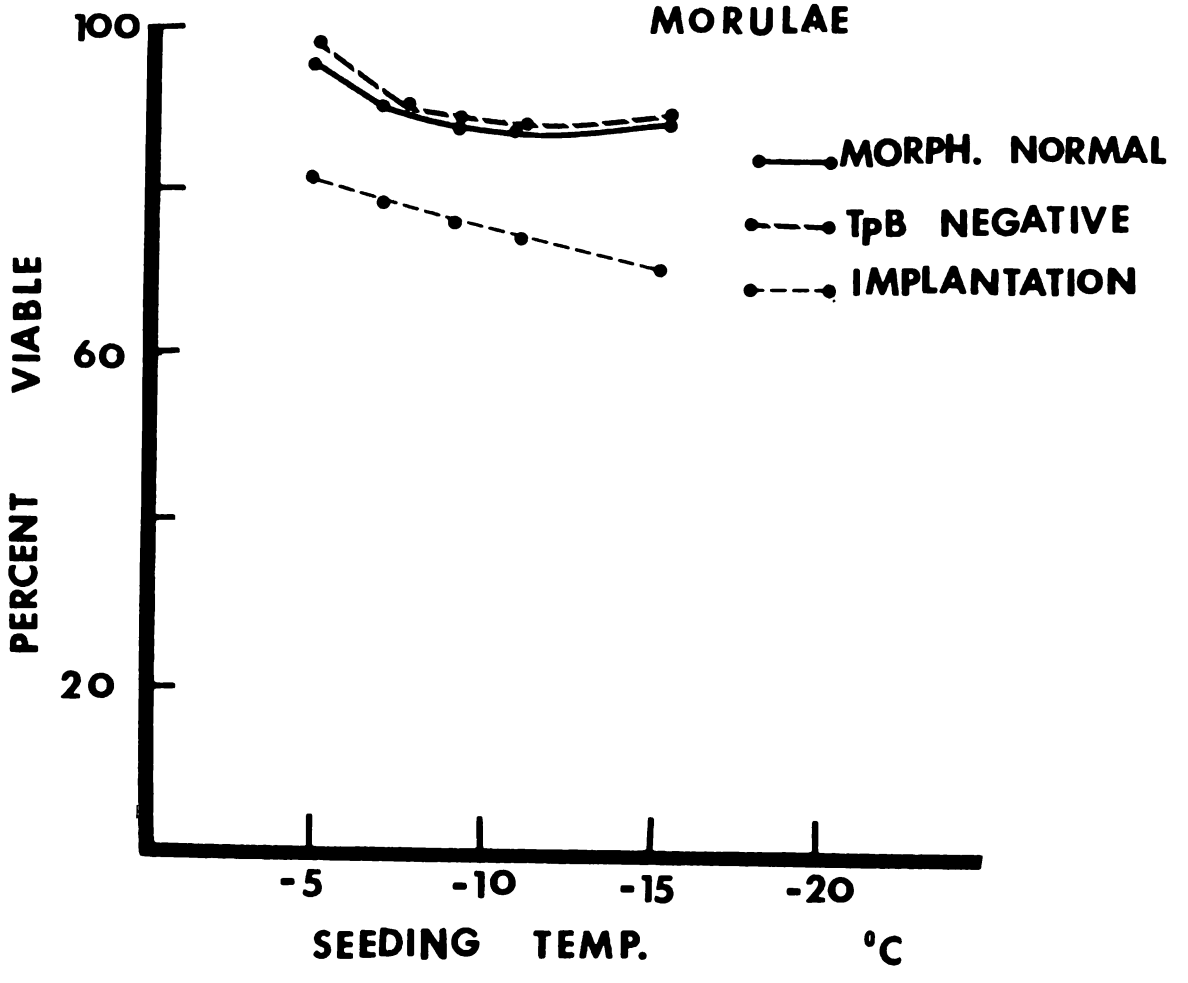


Figure 6. Survival of hamster morulae seeded at temperatures between -5°C and -15°C. Each point represents 2-3 replicates, ($p > .05$).

Morulae tolerated seeding temperatures up to -15°C without apparent loss in survival rate (Figure 6). The survivability of 1-, 2-, 4- and 8-cell embryos seeded at a temperature range of -5°C to -23°C (Figures 2, 3, 4 and 5 were similar. The normality and $TpB^-$ of morula were similar between seeding temperatures of -5 and -15°C. The number of implanted morulae decreased when the embryos were seeded below -15°C (Figure 6). The three tests of embryo viability were positively correlated (Figures 7, 8 and 9). The correlation coefficient between these tests was 0.99.

3. Freezing and Thawing Rates

There was no evidence ($p>.05$) that developmental stages of hamster embryos were adversely affected by slow cooling (0.33°C/min) and thawing (1.5°C/min) rates (Table 10). Of the 576 thawed embryos, 81% were normal morphologically and 84% by $TpB^-$. The viability of preimplantation embryos in terms of embryo normality and $TpB^-$ was significantly ($p<.001$) affected when these embryos were thawed at a rate of 90°C/min (Table 11). This thawing rate (90°C/min) did not affect significantly ($p>.05$) morulae and 8-cell embryos (Table 11). There was significant interaction between morulae and 1-, 2-, 4-cell embryos and also between 4-cell and 8-cell embryos ($p<.05$, Table 11) with respect to their normality and trypan blue reaction. These developmental stages showed higher sensitivity to rapid thawing ($p< .001$). The fertility of frozen-thawed embryos was not adversely affected by either slow or rapid thawing ($p>.05$, Tables 12 and 13). The viability of slow-thawed embryos was significantly higher ($p<.01$) than that of those rapidly thawed (Table 14). The number of pregnant recipients was not markedly different ($p>.05$) between slow and rapidly thawed embryos. The developmental potential

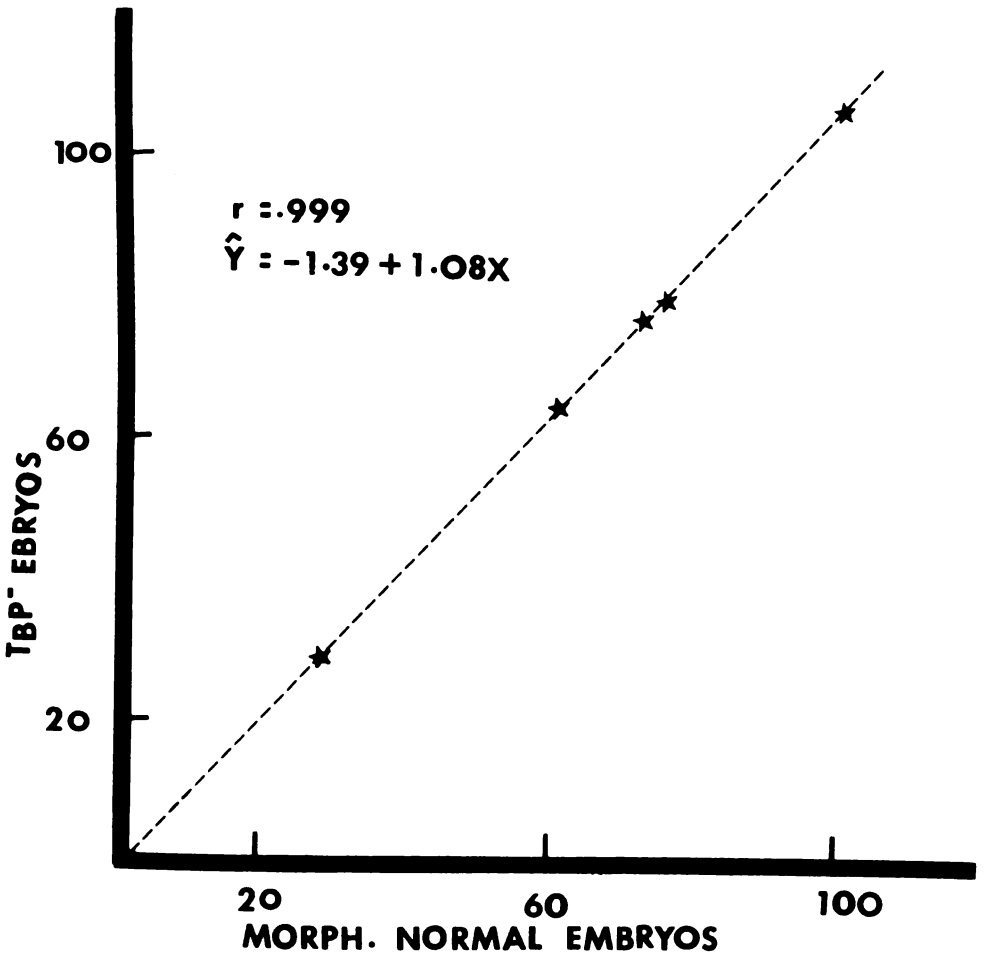


Figure 7. Positive correlation between $TpB^-$ and morphologically normal embryos. Each point represents 3-4 replicates.

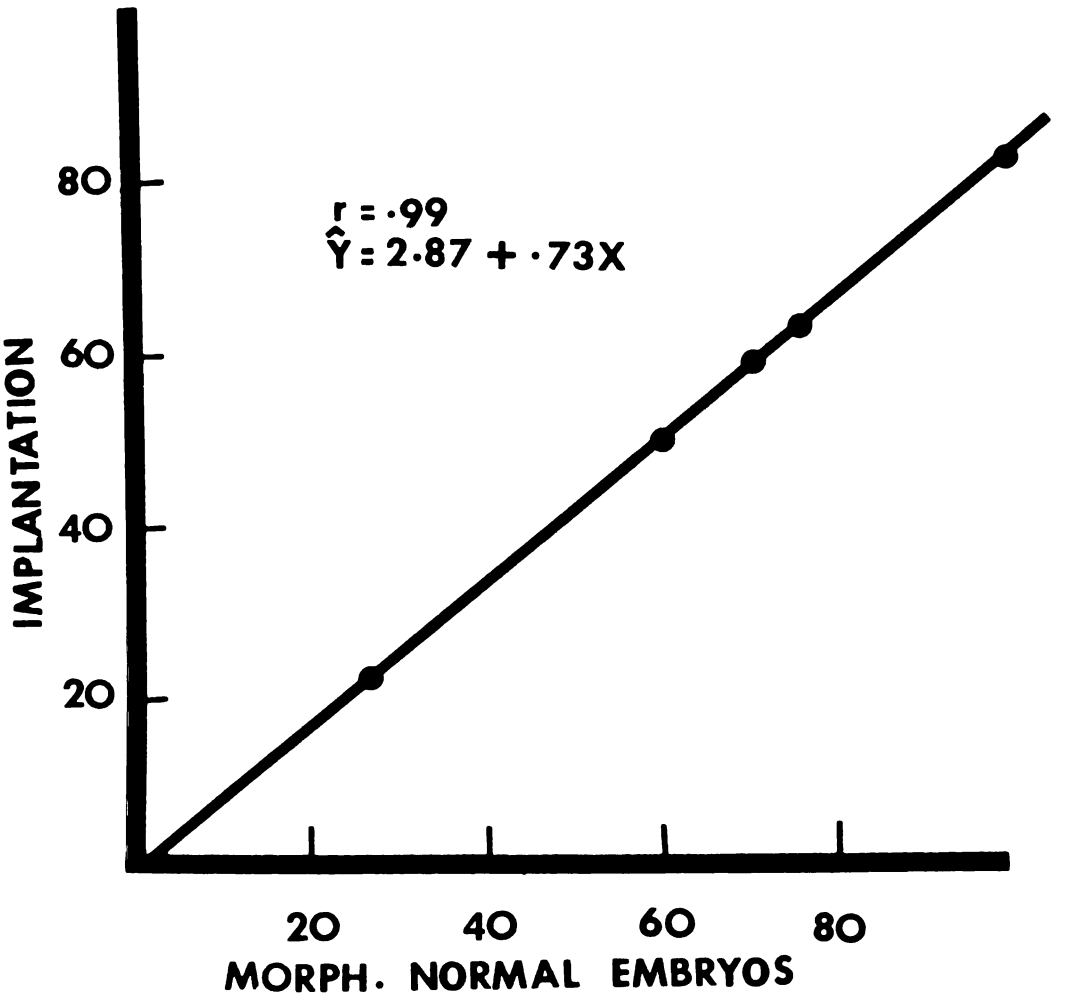


Figure 8. Positive correlation between morphologically normal embryos and implantation. Each point represents 3-4 replicates.

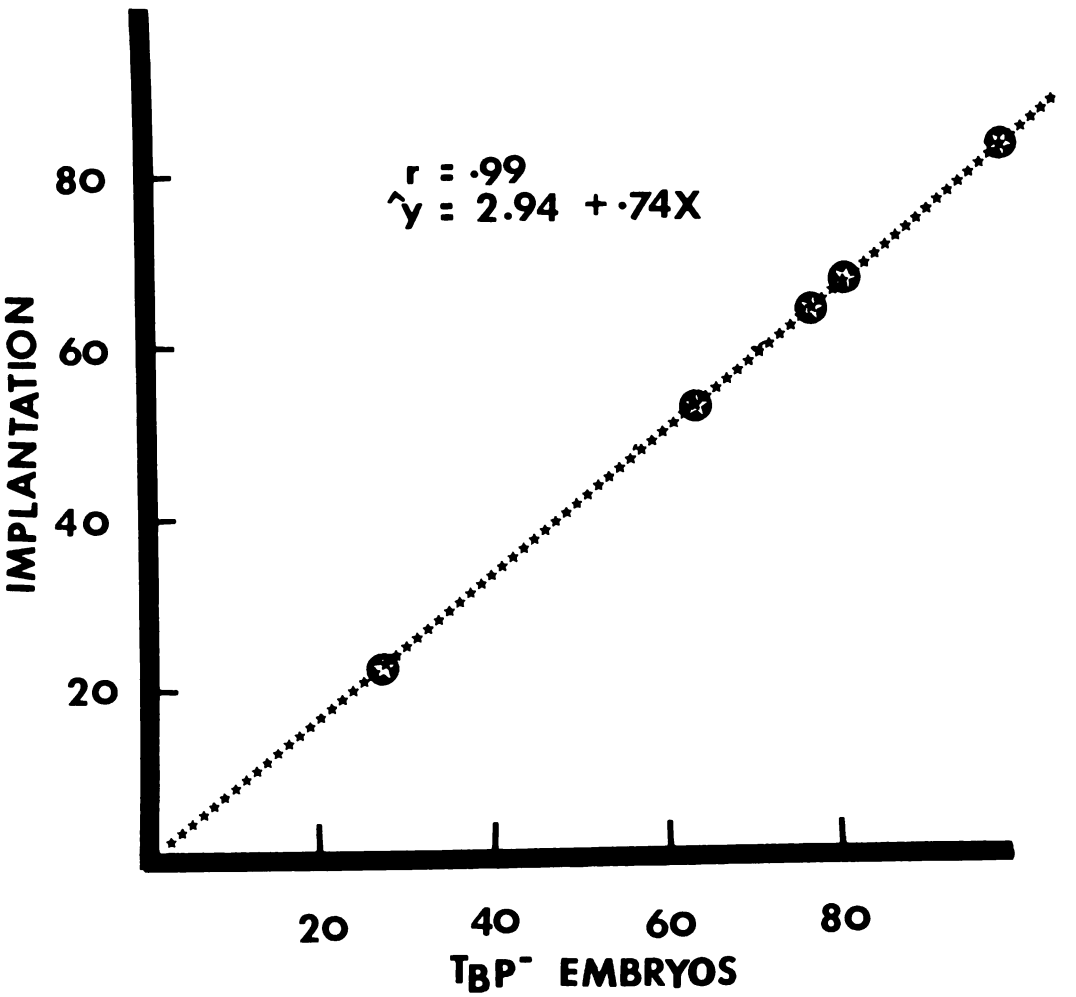


Figure 9. Positive correlation between $TpB^-$ embryos and implantation Each point represents 3-4 replicates.

TABLE 10

*Viability of Frozen Hamster Embryos Following Slow Thawing 1.5°C/min

| Cell Stage | Number Frozen | Number Thawed | Number Normal | Number $TpB^-$ (%) | Number Donors |
|---|---|---|---|---|---|
| 1-cell | 217 | 171 (79) | 141 (82) | 152 (89) | 15 |
| 2-cell | 196 | 156 (79) | 118 (76) | 133 (85) | 14 |
| 4-cell | 114 | 84 (74) | 69 (82) | 69 (82) | 11 |
| 8-cell | 101 | 63 (62) | 50 (79) | 52 (83) | 10 |
| Morula | 123 | 102 (83) | 86 (84) | 79 (77) | 12 |
| Total | 751 | 576 (77) | 464 (81) | 485 (84) | 62 |

* $p > .05$

TABLE 11

Viability of Frozen Hamster Embryos Following Rapid Thawing 90°C/min

| Cell Stage | Number Frozen | Number Thawed (%) | Number Normal (%) | Number $TpB^-$ (%) | Number Donors |
|---|---|---|---|---|---|
| 1-cell | 212 | 140 (66) | 94 (67)[a] | 90 (64)[b] | 14 |
| 2-cell | 228 | 193 (85) | 124 (64)[a] | 143 (74)[a] | 15 |
| 4-cell | 69 | 60 (87) | 30 (50)[b] | 32 (53)[b] | 6 |
| 8-cell | 71 | 46 (65) | 36 (78)[c] | 37 (80)[c] | 7 |
| Morula | 99 | 57 (58) | 49 (86) | 53 (93) | 9 |
| Total | 679 | 496 (73) | 333 (67)[d] | 355 (72)[d] | 51 |

a = $p < .05$ Significantly different from morula
b = $p < .01$ Significantly different from morula
c = $p < .05$ Significantly different from 4-cell
d = $p < .001$ Over all data

TABLE 12

*Fertility of Slowly Thawed Hamster Embryos Following Embryo Transfer

| Cell Stage | Number Transferred | Number Implanted (%) | Number Recipients | Number Pregnant (%) |
|---|---|---|---|---|
| 1-cell | 46 | 34 (74) | 6 | 5 (83) |
| 2-cell | 61 | 48 (79) | 8 | 7 (87) |
| 4-cell | 42 | 34 (81) | 8 | 7 (87) |
| 8-cell | 24 | 21 (87) | 5 | 5 (100) |
| Morula | 40 | 29 (73) | 7 | 6 (85) |
| Total | 213 | 166 (78) | 34 | 30 (88) |

* $p > .05$

TABLE 13

*Fertility of Rapidly Thawed Hamster Embryos Following Embryo Transfer

| Cell Stage | Number Transferred | Number Implanted (%) | Number Recipients | Number Pregnant (%) |
|---|---|---|---|---|
| 1-cell | 42 | 33 (78) | 6 | 5 |
| 2-cell | 58 | 39 (67) | 6 | 4 |
| 4-cell | 16 | 11 (69) | 3 | 2 |
| 8-cell | 22 | 18 (82) | 4 | 4 |
| Morula | 28 | 16 (57) | 4 | 3 |
| Total | 166 | 117 (70) | 23 | 18 (78) |

* $p > .05$

TABLE 14

Fertility of Frozen Hamster Preimplantation Embryos
Following Slow and Rapid Thawing Procedures

| Thawing Rate | Number Frozen | Number Thawed (%) | Number Normal (%) | Number $TpB^-$ (%) | Number Transferred | Number Implanted (%) | Number Recipients | Number Pregnant (%) |
|---|---|---|---|---|---|---|---|---|
| 1.5°C/min | 751 | 576 (77) | 464 (81)[a] | 485 (84)[b] | 213 | 166 (78) | 34 | 30 (88) |
| 90°C/min | 679 | 496 (73) | 333 (67) | 355 (72) | 166 | 117 (70) | 23 | 18 (78) |
| Total | 1430 | 1072 (75) | 797 (74) | 840 (78) | 379 | 283 (75) | 57 | 48 (84) |

a = $p < .001$ Significantly greater than those rapidly thawed

b = $p < .01$ Significantly greater than those rapidly thawed

TABLE 15

The Developmental Potential of Slowly Cooled and Thawed Hamster Embryos in Either PBS or TC-199 Containing 1.5M-DMSO

| Embryo Stage | Number Transferred | Number Implanted* (%) | Number fetuses/ Number implanted** (%) | Number fetuses/ Number transferred (%) |
|---|---|---|---|---|
| 1-cell | 50 | 37 (74) | 8 [a,b](22) | [a,b](16) |
| 2-cell | 90 | 70 (78) | 29 [a,c](41) | [a,c](32) |
| 4-cell | 28 | 23 (82) | 16 (70) | (57) |
| 8-cell | 49 | 41 (84) | 30 (73) | (61) |
| Morula | 35 | 28 (80) | 22 (78) | (63) |
| Total | 252 | 199 (79) | 105 [d](53) | [d](41) |

a = $p < .01$ Significantly different from morula
b = $p < .01$ Significantly different from 4- and 8-cell embryos
c = $p < .01$ Significantly different from 8-cell embryos
d = $p < .001$ Total data: Developmental stages of the embryos vs. developed fetuses

* Day 6 of pregnancy
** Day 14 of pregnancy

of slowly cooled and thawed hamster preimplantation embryos on day 14 of pregnancy was significantly ($p<.001$) affected by the developmental stages of the embryo (Table 15). Small numbers of 1-cell and 2-cell embryos were developed to day 14 fetuses compared to 4-, 8-cell and morula (Table 15).

B. Embryo Transfer

The transfer of 893 unfrozen embryos from 204 donors to 216 recipients resulted in 60% implantation (Table 16). There were no significant differences between developmental stages and implantation rates. The pregnancy rate of unfrozen embryos was higher for all transferred preimplantation embryos (87%, Table 17). No significant interactions were observed between developmental stages of the embryos and pregnancy rates. The recovery times of superovulated embryos were longer (9 h delay) than for naturally ovulated embryos ($p<.001$, Table 18). There were significant differences between the recovery times and the developmental stages of the embryos ($p<.05$). The implantation rates were significantly lower ($p< .005$) in the superovulated group compared to natural ovulation (Table 19). Higher implantation rates were observed in naturally ovulated 1-cell embryos (71%) compared to superovulated 1-cell embryos (54%, $p<.05$). The transfer of 1-cell or 2-cell embryos either I.B. or I.O. gave similar results (Table 20). The intrauterine transfer of 4-cell and 8-cell embryos was more effective ($p<.05$) than the intrabursal transfer of the same developmental stages of the embryo (Table 21). Bilateral transfer of preimplantation embryos showed lower implantation rates (55%) than that of unilateral transfer (62%, $p<.1$). The developmental stages of the embryos were not significantly affected

TABLE 16

*Implantation Following Unfrozen Embryo Transfer in Golden Hamster

| Cell Stage | Number Exp. | Number Donor | Number Recip. | Number Transf. | Number Implan. | Implan. Percent |
|---|---|---|---|---|---|---|
| 1-cell | 13 | 52 | 57 | 298 | 164 | 55 |
| 2-cell | 23 | 102 | 103 | 343 | 211 | 61 |
| 4-cell | 7 | 18 | 24 | 77 | 46 | 64 |
| 8-cell | 9 | 32 | 32 | 175 | 112 | 60 |
| Total | 52 | 204 | 216 | 893 | 533 | 60 |

* $p > .05$

TABLE 17

*Pregnancy Percentage in Golden Hamster Following Embryo Transfer

| Cell Stage | No. Transf. Embryos | No. Recip. | No. Pregn. Recip. | Pregn. % |
|---|---|---|---|---|
| 1-cell | 298 | 57 | 48 | 84 |
| 2-cell | 343 | 103 | 95 | 92 |
| 4-cell | 77 | 24 | 19 | 79 |
| 8-cell | 175 | 32 | 26 | 81 |
| Total | 893 | 216 | 188 | 87 |

* $p > .05$

TABLE 18

Recovery Times of Hamster Embryos

| Cell Stage | Natural Ovulation Hour after mating Mean | (Range) | Superovulation Hour after mating Mean | (Range) |
|---|---|---|---|---|
| 1-cell | [a]26 | (24-37) | 31 | (18-50) |
| 2-cell | [a]36 | (25.5-53) | 43.14 | (24-64) |
| 4-cell | [a]51.51 | (51-53.5) | 69.6 | (65-74) |
| 8-cell | [a]68.7 | (64-76) | 72.52 | (68-76) |
| Total | [b]45.5 | (24-76) | 54.06 | (18-76) |

a = $p < .05$ Significantly different from corresponding superovulated embryos

b = $p < .001$ Overall data

TABLE 19

Survival of Superovulated Hamster Embryos Following Embryo Transfer

| Cell Stage | Natural Ovulation No. implanted/No. transferred (%) | Superovulation* No. implanted/No. transferred (%) |
|---|---|---|
| 1-cell | 30/42 [a](71) | 147/273 (54) |
| 2-cell | 57/85 (67) | 154/258 (60) |
| 4-cell | 26/39 (67) | 20/38 (53) |
| 8-cell | 37/55 (67) | 75/120 (62) |
| Total | 167/246 [b](68) | 396/689 (57) |

* Both groups (donor and recipient) were superovulated

a = $p < .05$ Significantly different from superovulated 1-cell embryos

b = $p < .005$ Overall data

TABLE 20

*Implantation in Golden Hamster Following Intrabursal and Intraoviductal Transfer of Preimplantation Embryos

| Cell Stage | Intrabursal Transfer No. Implanted/No. Transferred (%) | | Intraoviductal Transfer No. Implanted/ No. Transferred (%) | |
|---|---|---|---|---|
| 1-cell | 152/277 | (55) | 23/41 | (56) |
| 2-cell | 184/299 | (61) | 27/44 | (61) |
| Total | 336/576 | (58) | 50/85 | (59) |

* $p > .05$

TABLE 21

Implantation in Golden Hamster Following Intrabursal and Intrauterine Transfer

| Cell Stage | Intrabursal Transfer No. implanted/No. transferred (%) | | Intrauterine Transfer No. implanted/No. transferred (%) | |
|---|---|---|---|---|
| 4-cell | 21/39 | (54) | 25/38 | (66) |
| 8-cell | 25/47 | (53) | 87/128 | (68) |
| Total | 46/86 | (53) | 112/166 | [a](67) |

a = $p < .05$ Significantly different from intrabursal transfer

TABLE 22

Effect of Side of Embryo Transfer on Implantation in Golden Hamster

| Cell Stage | Unilateral Transfer No. implanted/No. transferred (%) | | Bilateral Transfer No. implanted/No. transferred (%) | |
|---|---|---|---|---|
| 1-cell | 121/206 | [a](59) | 43/92 | (47) |
| 2-cell | 123/195 | (63) | 88/148 | (59) |
| 4-cell | 33/55 | (60) | 19/32 | (59) |
| 8-cell | 81/121 | (67) | 31/54 | (57) |
| Total | 358/577 | [b](62) | 181/326 | (55) |

a = $p < .1$ Significantly different from Bilateral Transfer
b = $p < .1$ Overall data

TABLE 23

Effect of Uterine Horn on Implantation of
Golden Hamster Embryos

| Cell Stage | Right Horn<br>No. implanted/No. transferred<br>(%) | Left Horn<br>No. implanted/No. Transferred<br>(%) |
|---|---|---|
| 1-cell | 84/166 (51) | [a]80/132 (61) |
| 2-cell | 107/84 (58) | 104/159 (65) |
| 4-cell | 29/46 (63) | 17/31 (55) |
| 8-cell | 53/78 (68) | 59/97 (61) |
| Total | 273/474 (58) | 260/419 (62) |

a = $p < .05$ Significantly different from right horn

($p>.05$) by the type of transfer (Table 22). The transfer of 1-cell embryos to the left horn yield higher implantation rates ($p<.05$) compared to the right horn (Table 23). Both horns were equally effective for the transfer of other embryo stages (Table 23). Two transfer media (PBS and TC-199) were used for embryo transplantation. Both media were found to be effective except in 8-cell embryos ($p<.01$) where the implantation rate was higher in TC-199 than PBS (Table 24). The implantation rate of preimplantation embryos obtained either from young (14 wk) or mature (26 wk) hamsters did not differ significantly ($p>.05$, Table 25). The numbers of transferred embryos had no significant effect ($p>.05$) on the implantation rates of the developmental stages of the embryos (Table 26). The transfer of 7 to 9 embryos vs. 3 to 6 or 10 to 12 embryos to recipient females showed relatively higher implantation rate than the other two groups (66% vs. 61% and 57% respectively).

Embryo migration was determined by unilateral transfer of preimplantation embryos into one uterine horn. The different developmental stages showed significantly ($p<.05$) different migratory capability (Table 27). One-cell embryos had a higher migration rate ($p<.05$) than the two-cell embryos. Migration of 1-cell embryos to the left uterine horn was significantly higher ($p< .05$) than to the right uterine horn.

TABLE 24

Viability of Hamster Embryos in Two Transfer Media (PBS vs. TC-199)

| Embryo Stage | PBS No. Implanted/ No. Transferred (%) | | TC-199 No. Implanted/ No. Transferred (%) | | Number Recipients |
|---|---|---|---|---|---|
| 1-cell | 78/140 | (56) | 86/158 | (54) | 57 |
| 2-cell | 79/133 | (59) | 132/210 | (63) | 103 |
| 4-cell | 17/32 | (53) | 29/45 | (64) | 24 |
| 8-cell | 41/77 | (53) | 71/98 | [a](72) | 32 |
| Total | 215/382 | (56) | 318/511 | (62) | 216 |

a = $p < .01$ Significantly different from PBS medium

TABLE 25

Implantation of Hamster Embryos in Young and Mature Recipients Following Embryo Transfer

| Cell Stage | Young Hamster[a] No. Implanted/ No. Transferred (%) | | Mature Hamster[b] No. Implanted/ No. Transferred (%) | |
|---|---|---|---|---|
| 1-cell | 100/189 | (53) | 64/109 | (59) |
| 2-cell | 101/170 | (59) | 110/173 | (53) |
| 4-cell | 29/50 | (58) | 17/27 | (63) |
| 8-cell | 69/111 | (62) | 43/64 | (67) |
| Total | 299/520 | (57) | 234/373 | (63) |

a = Age range 12-16 weeks, mean: 14 weeks, young group
b = Age range 24-28 weeks, mean: 26 weeks, mature group

TABLE 26

Receptivity of Uterine Environment to the Number of Transferred Embryos Following Embryo Transfer in Golden Hamster

| Cell Stage | 3 - 6 Embryos | | 7 - 9 Embryos | | 10 - 12 Embryos | |
|---|---|---|---|---|---|---|
| | No. Implanted/ No. Transferred | (%) | No. Implanted/ No. Transferred | (%) | No. Implanted/ No. Transferred | (%) |
| 1-cell | 29/52 | (56) | 49/79 | (62) | 55/95 | (58) |
| 2-cell | 73/114 | (64) | 88/136 | (65) | 44/79 | (56) |
| 4-cell | 16/29 | (55) | 25/38 | (66) | 14/24 | (58) |
| 8-cell | 56/91 | (61) | 43/59 | (73) | 13/22 | (59) |
| Total | 174/286 | (61) | 205/312 | (66) | 126/220 | (57) |

TABLE 27

Embryo Migration in Golden Hamster Following Embryo Transfer

| Cell Stage | No. Embryos Transferred to Left Horn | No. Embryos Migrated to Right Horn (%) | No. Embryos Transferred to Right Horn | No. Embryos Migrated to Left Horn (%) | Total Migration No. migrated/ No. transferred (%) |
|---|---|---|---|---|---|
| 1-cell | 131 | 11 (8) | 177 | 32 [c](18) | 43/308 [a](14) |
| 2-cell | 153 | 9 (6) | 190 | 17 ( 9) | 26/343 ( 7) |
| 4-cell | 31 | 2 (6) | 46 | 3 ( 6) | 5/77 ( 6) |
| 8-cell | 97 | 13 (13) | 78 | 8 (10) | 21/175 (12) |
| Total | 412 | 35 (8) | 491 | 60 (12) | 95/903 [b](10) |

a = $p < .05$ Significantly different from 2-cell
b = $p < .05$ Migratory capability vs. developmental stages of the embryos
c = $p < .05$ Migration: left vs. right

# DISCUSSION

The survival of frozen-thawed hamster preimplantation embryos was highest in TC-199 medium compared to PBS medium. The 1- and 2-cell embryos showed higher sensitivity to PBS compared to morulae, whereas no such observation was noticed in TC-199 medium. The low survival rates of hamster embryos in PBS medium could be due to the adverse effect of the pH. The optimum pH for hamster embryos was found to be 7.2 - 7.4 (Whittingham and Bavister, 1974; Whittingham, 1979). PBS medium is detrimental to the survival of mouse embryos at 37°C (Quinn and Wales, 1973). In the present experiments PBS was not supplemented with an external energy source. Therefore, the lack of protein and glucose in the PBS may also be responsible for the low survival rate. The results of the present experiments agree with those of Fleming et al. (1979) and Quinn et al. (1982). The later investigators attributed the low survival rates of hamster oocytes in PBS to the low protein concentrations and the buffering system. Tsunoda and Sugie (1977a) reported that the addition of rabbit serum to PBS resulted in a significant increase in the developmental rate of rabbit embryos and also in the number of newborn compared to PBS alone. One-cell and two-cell hamster embryos are highly affected by culture media (Sato and Yanagimachi, 1972; Whittingham and Bavister, 1974; and Whittingham, 1978) and the results of the present study support this. The fertility of frozen-thawed hamster embryos was similar in both media and this indicates that the developmental stages of the hamster embryos were not affected by the freezing media. Similar results were obtained with mouse embryos when PBS was used as a freezing medium (Whittingham et

al., 1972; Wilmut, 1972). The survival rates of early preimplantation embryos (following freezing and thawing) were markedly decreased when these embryos were seeded below -10°C, whereas the late embryonic stage was unaffected even when seeded at -15°C. These results demonstrate that the early preimplantation embryos are highly sensitive to seeding temperature and this confirms similar observations with mice (Whittingham, 1977a; Miyamoto and Ishibashi, 1981a) and sheep embryos (Moore and Bilton, 1977). The higher tolerance of hamster morulae to the seeding temperature probably reflects the ability of morulae to undergo sufficient dehydration during freezing at the time when intracellular freezing takes place. Rall et al. (1983) observed that the nucleation temperature was responsible for the drastic decrease in the survival of mouse embryos. Supercooling of mouse embryos below -7°C significantly decreased survival rate and when mouse morulae were seeded below -10°C there was a lower survival rate (Whittingham, 1977a; Miyamoto and Ishibashi, 1981a). These findings are inconsistent with regard to the observations with hamster 8-cell and morulae in the present study. This discrepancy could be due to species specificity to seeding and also to the methods of seeding induction. The implantation rates of 1- to 8-cell stages were significantly different from the other tests at seeding temperatures below -11°C. This finding suggests that implantation is not dependent or related to the other tests. The three tests of survival were positively correlated at seeding temperatures above -10°C.

The viability and fertility of various preimplantation embryos relative to slow cooling and thawing were similar. This means that all the preimplantation embryos tolerate equally slow freezing and thawing by adequate dehydration. These results agree with those of Tsunoda

et al. (1976); Parkening and Chang (1977); and Quinn et al. (1982) who reported similar results with hamster oocytes following slow cooling and thawing. Rapid thawing after slow freezing significantly lowered the survival of 1- to 4-cell hamster embryos in the present experiments. Similar results were reported for mouse (Whittingham et al., 1972; Wilmut, 1972; Liebo et al., 1974), sheep (Willadsen, 1976) and cow (Wilmut and Rowson, 1973) embryos. The lower survival rates of hamster embryos after rapid thawing confirm previous findings of Leibo et al. (1974 and 1977). These results also support other investigators who found that rapid thawing is more detrimental to embryo survival than slow thawing (Whittingham et al., 1972; Wilmut, 1972; Bank, 1973: Leibo et al., 1974; Farrant, 1977). It seems that cell membranes at lower temperatures are less resistant to deformation produced by osmotic changes and rapid thawing may cause osmotic shock to hamster embryos. The results of the present study support the earlier suggestion of Bank (1973) and Farrant (1977) who recommended slow thawing for slowly cooled embryos to avoid cell damages by osmotic shock.

The highest sensitivity to development to day 6 and 14 fetuses was observed in 1- and 2-cell hamster embryos. The pre- and post-implantation losses of these embryos were 24% and 68% respectively. These results are in contrast to those of Whittingham et al. (1972) who found that 1- and 2-cell mouse embryos were less sensitive to freezing damage than blastocysts. These differences may be due to species sensitivity to freezing and this was reported by several workers (Tsunoda et al., 1976; Maurer, 1976; Parkening and Chang, 1977; and Whittingham, 1978 and 1979). The developmental potential of early hamster embryos following slow freezing and thawing agrees with those of

Tsunoda and Sugie (1977b) who found lower developmental rates in 2-cell rabbit embryos after freezing. The marked reduction in the developmental potential of early hamster embryos could be because these embryos were not cultured *in vitro* after thawing but immediately transferred to recipients. This finding confirms that of Whittingham and Anderson (1976) and Whittingham (1977a) who showed that immediate transfer of mouse embryos after thawing without culturing resulted in a significant decrease in survival rate. These workers also reported that mouse embryos demonstrate developmental delay after freezing and in order to resume normal embryonic development a restorative period in culture is required.

The pregnancy and implantation rates of unfrozen hamster embryos were similar, which indicates that there are no specific interactions between the development stages of hamster embryos and their ability to implant. The uterine environment seems to have no selective effect on the various developmental stages of hamster embryos. Kusanagi (1981) found that embryo transfer had no effect on the developmental capability of mouse embryos. The results of the present experiments agree with those of Ghosh *et al.* (1982). Similar results also were obtained in mice following embryo transfer (Moler *et al.*, 1979). However, the findings of the present experiments are in contrast to those of Sato and Yanagimachi (1972) who reported lower embryonic development in hamsters after embryo transfer. These differences may be due to modification of the transfer medium and techniques in the present study.

Superovulation regimens induced a 9-hour delay in ovulation and also significantly lowered implantation rates compared to naturally ovulating embryos. These observations confirm those of previous workers who

reported that superovulation produced side effects in rodents (Fujimoto et al., 1974; Takagi and Sasaki, 1976; Maudlin and Fraser, 1977; Mizoguchi and Dukelow, 1981). Similar treatment in hamsters induces unilateral pregnancy and alteration in ovulation and embryo transport (Fleming and Yanagimachi, 1980). The lower implantation rates in superovulated embryos could be due to the effect of hormones on the embryo itself (Katzberg and Hendrickx, 1966; Shaver and Carr, 1967; Shaver, 1970; Fujimoto et al., 1974; Takagi and Sasaki, 1976) and/or the uterine environment of the superovulated recipient (Yang and Chang, 1968; Banik, 1975, Betteridge, 1977). Similar ovulation delay was also reported in golden hamster embryos by Ghosh et al. (1982).

The I.B. and I.O. transfer of 1- and 2- cell embryos did not show differences in implantation rates; however, the I.B. transfer of 4- and 8-cell hamster embryos resulted in a marked decrease in implantation rate compared to I.O. transfer. The lower implantation rate of I.B. transfers could be due to the adverse environment of the ovarian bursa which might cause a delay in embryo transport and subsequently result in aged embryos. It has been shown that the normal site for 4-cell and 8-cell embryos is the oviduct or uterus (Sato and Yanagimachi, 1972; Whittingham, 1979: Ghosh et al., 1982). One-cell and 2-cell mouse and rat embryos undergo degeneration when transferred to the uterus (Noyes et al., 1963), whereas they develop normally after transfer to the oviduct (Tarkowski, 1959; Whittingham, 1979).

The unilateral transfer of hamster embryos yielded higher implantation rates than bilateral transfer. This may be due to the longer surgical stress and recovery period with bilateral transfer than unilateral transfer. McLaren (1970) showed that an empty uterine horn

does not exert a systemic or local luteolytic effect on the survival of mouse eggs transferred to the other horn, whereas Adams (1962) reported that in rabbits the type of transfer had no effect on egg survival after transfer. These observations are consistent with results of the present experiment; however, Muller and Carter (1973) in mice, and Tsunoda et al. (1982) in rabbits, observed a higher survival rate of embryos transferred bilaterally. This discrepancy may be due to species and also variation in transfer technique.

The viability of preimplantation hamster embryos in transfer media was similar except that 8-cell embryos showed a higher sensitivity to PBS than TC-199 medium. The same explanations for PBS as a freezing medium are valid for PBS as a transfer medium for hamster embryos.

It is well known that the various developmental stages of embryos demonstrate different responses to the same medium or different media (Whittingham and Bavister, 1974; Hanada and Chang, 1976; Whittingham, 1979). Hamster oocytes and embryos are found whether fertilized in vivo or in vitro to be highly sensitive to culture media, especially to the pH (Hanada and Chang, 1976; Whittingham, 1979). It was indicated from the results of the present experiments that equivalent age of the donors and recipient has no effect on implantation rate of hamster embryos following embryo transfer. The relative higher survival of hamster preimplantation embryos following embryo transfer which was observed in the mature group rather than the young group may reflect the increased maturation in the 26-week-old hamster. These results agree with those of Mizoguchi and Dukelow (1981) and Evans and Dukelow (1982) who found an increase in fertility with older hamsters.

The various numbers of the transferred embryos did not show a significant interaction with respect to their implantation in the recipient hamsters. The lower implantation rate in the 10 - 12 transferred embryo group may be due to the spacing phenomenon, which could act as a stress factor leading to preimplantation mortality. These results are consistent with those of Sato and Yanagimachi (1972) who obtained similar implantation rates after deposition of 3 to 5 embryos per horn. Similar implantation rates were observed in mice, rats and hamsters after the deposition of 5 to 8 embryos (Humphrey, 1967; Toyada and Chang, 1974; Fiser and Macpherson, 1982), and this confirms the results of the present study.

A 10% embryo migration was observed in hamsters following the transfer of 903 embryos unilaterally. The highest migration was observed in 1-cell embryos. The migration of preimplantation hamster embryos may be due to production of estradiol by the embryos which causes uterine contraction and increases embryo migration. This conclusion is supported by Pope et al. (1982a) who found in pigs that uterine contraction increases with embryo migration and the factor responsible for uterine contraction was hormonal in nature. Ghosh et al. (1982) attributed the higher uptake of estradiol in the uterine tissue following 8-cell embryo transfer due to the increased production of estradiol by the 8-cell embryo. This supports the higher migratory percentage of the 8-cell embryos than the 2-cell and 4-cell embryos in the present study. The production of estradiol from hamster embryos has been suggested by several investigators (Dickmann and Sengupta, 1974; Dickmann et al., 1976; and Sengupta, et al., 1981). Dickmann and Sengupta (1974) reported that hamster embryos contain

$\Delta^5$-3β-hydroxysteroid dehydrogenase and 17-β-hydroxysteroid dehydrogenase, which are both involved in biosynthesis of steroid hormones. Other workers observed that estradiol-17 directly affects the transport of mouse embryos and also has synergistic action with progesterone on embryo transport and development (Roblero and Garavagno, 1979). These findings are consistent with the stimulatory effect of embryonic estradiol on embryo migration in the hamster. Another factor which might be responsible for the migration of hamster preimplantation embryos is the spacing phenomenon since higher migration was observed with the deposition of larger number of embryos (10 - 16 embryos) in the recipient females.

# SUMMARY AND CONCLUSIONS

The golden hamster (Mesocricetus auratus) was studied as a model for embryo production, freezing and transfer. Hamster preimplantation embryos (1-cell to morula) were recovered after either natural ovulation or superovulation. Survival of hamster embryos in different freezing media, seeding temperatures, freezing and thawing rates was evaluated by morphological appearance, trypan blue exclusion and embryo transfer techniques. The developmental potential of slowly cooled and thawed embryos was also examined by transfer of these embryos into synchronized recipient females. Pregnancy and implantation rates of various developmental stages of unfrozen hamster embryos and their interactions with different factors were determined. The following conclusions were obtained from the results of these studies:

1. TC-199 was superior to PBS as a freezing medium. The 1- and 2- cell embryos were adversely affected by PBS medium.

2. The early preimplantation embryos are highly sensitive to seeding temperature, whereas late embryos tolerate temperatures as low as -15°C without apparent loss in viability. Optimal seeding temperature for hamster embryos was found to be above -9°C.

3. Higher survival rates for hamster preimplantation embryos were found with slow thawing (1.5°C/min) after slow cooling (0.33°C/min).

4. The developmental potential of hamster embryos showed significant interaction with the developing stages of the embryos. The early preimplantation embryos were more susceptible to freezing damage.

5. Superovulated embryos showed a 9 h delay in ovulation and had lower implantation rates than naturally ovulated embryos.

6. One-cell and 2-cell embryos should be transferred I.O. or I.B. but other embryos should be deposited in the uterus. The site of transfer was found to affect significantly the developmental stages of the embryo.

7. No significant effects were observed on the survival of unfrozen embryos when placed in uterine horns and by the side of transfer.

8. TC-199 as transfer medium is superior to PBS.

9. The transfer of 3 to 9 embryos per side from mature hamsters (24 to 28 weeks old) is recommended.

10. Embryo migration was found in the hamsters and 1-cell embryos showed the highest migratory capability.

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# APPENDIX A

# APPENDIX A

## TABLE 29

*Viability of Frozen-Thawed Cow Eggs Following Seeding at Different Temperatures

| Number Frozen | Number Thawed (%) | Number Normal (%) | Number $TpB^-$ (%) | Period Frozen | Seeding Temperature |
|---|---|---|---|---|---|
| 48 | 46 (96) | 34 (74) | 39 (85) | 17h | -5°C |
| 41 | 34 (83) | 29 (85) | 27 (79) | 2d | -6°C |
| 57 | 48 (84) | 42 (88) | 43 (90) | 2d | -7°C |
| 92 | 89 (97) | 85 (96) | 85 (96) | 17d | -8°C |
| 238 | 217 (91) | 190 (88) | 194 (89) | 17h-17d | All Temperatures |

* r = 0.99 Morphologically normal embryos and $TpB^-$ Embryos

Cooling rate = 0.25°C/min
Thawing rate = 0.50°C/min
Freezing medium = TC-199 and PBS

# APPENDIX A

## TABLE 28

*Effect of Freezing Medium on Viability of Cow Eggs

| Number Frozen | Number Thawed (%) | Number Normal (%) | Number $TpB^-$ (%) | Period Frozen | Freezing Medium |
|---|---|---|---|---|---|
| 66 | 57 (86) | 53 (93) | 55 (96) | 4h-12d | PBS |
| 139 | 130 (93) | 110 (85) | 115 (88) | 17h-17d | TC-199 |
| 52 | 43 (83) | 37 (86) | 36 (84) | 30h-17d | DMEM |
| 257 | 230 (89) | 200 (87) | 206 (90) | 4h-17d | All Media |

* r = .999 Morphologically normal embryos and $TpB^-$ embryos

Slow cooling rate = 0.25°C/min
Slow thawing rate = 0.50°C/min
Seeding temperature = -7°C
Cow follicular oocytes

# APPENDIX B

# APPENDIX B

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11. Survival of cow follicular oocytes frozen at -196°C. (in preparation)

12. Deep double freezing of cow oocytes and hamster eggs and embryos. (in preparation)

# APPENDIX C

# APPENDIX C

## CURRICULUM VITAE

| | |
|---|---|
| Name: | MUNDHIR TAIYEB RIDHA |
| Born: | July 16, 1949 |
| Birthplace: | Kanakin, Iraq |
| Formal Education: | Kanakin High School |
| | College of Veterinary Medicine<br>University of Baghdad<br>Baghdad, Iraq |
| Degrees Received: | Bachelor of Veterinary Medicine and Surgery<br>College of Veterinary Medicine<br>University of Baghdad, 1974 |
| | Master of Science<br>College of Veterinary Medicine<br>University of Baghdad, 1976 |
| Experience: | Assistant Lecturer and Laboratory Supervisor in Physiology, Histology and Embryology |
| | Research Scientist<br>University of Baghdad<br>Department of Physiology and Biochemistry |
| | University of Basrah<br>Department of Biology and Chemistry<br>1977-1979 |
| Membership in: | Society for the Study of Reproduction |
| | American Society of Animal Science |
| | American Association for the Advancement of Science |
| | International Embryo Transfer Society |
| | Iraqi Veterinary Medical Association |