APPLICATION OF INSECT FREEZE TOLERANT STRATEGIES TO THE FREEZING OF BOVINE EMBRYOS

by

Sarah S. Whitman

Thesis submitted to the Faculty of the

Virginia Polytechnic Institute and State University

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Dairy Science

APPROVED:

Dr. R.G. Saacke, Chairman

Dr. J.A. Lineweaver

Dr. D.E. Mullins

Dr. R.E. Pearson

Dr. J.M. White, Department Head

July, 1984
Blacksburg, Virginia

APPLICATION OF INSECT FREEZE TOLERANT STRATEGIES TO THE FREEZING OF BOVINE EMBRYOS

by

Sarah S. Whitman

(ABSTRACT)

Hemolymph of Tipula trivittata larvae permits freeze tolerance of this insect due to its content of cryoprotectants and ice nucleating proteins. Spontaneous ice nucleation of the dialyzed hemolymph occurs between -5 C and -11 C up to dilutions of 1:1000. The objectives of this study were to evaluate the effects of seeding temperature (-5 C vs -7 C), and the presence of hemolymph at a low (.1% v/v) and a high (10% v/v) level on the survival of frozen-thawed bovine embryos. In Exp. 1, survival rates of 6 and 7 day bovine embryos frozen in medium containing .1% hemolymph and seeded at -5 C or -7 C, were compared to evaluate the effect of seeding temperature. The effect of hemolymph was evaluated by including a control without hemolymph seeded at -7 C. In Exp. 2, survival rates of embryos frozen with and without 10% hemolymph were compared. In Exp. 3 the evaluation of the effect of 10% hemolymph was continued. Also included was a control handled identically to embryos frozen

in medium with 10% hemolymph regarding pre and post freeze manipulations but which was not frozen. This allowed evaluation of freezing damage per se. For Exps. 1, 2, and 3, survival based on mean final development score and time to advance a developmental stage in vitro did not differ for embryos frozen. However, in Exp. 3, the control which was not frozen had 30% greater survival than embryos undergoing the same manipulations but which were frozen. Thus, neither seeding temperature nor inclusion of .1% or 10% hemolymph in freezing medium had a significant effect on survival of frozen-thawed bovine embryos.

ACKNOWLEDGEMENTS

Sincere appreciation is extended to the following people:

Very special thanks to Dr. R. G. Saacke, my major professor for his patient guidance, his objective and stimulating criticism, and his wonderful enthusiasm throughout my graduate studies.

Dr. J. A. Lineweaver for his generosity in the provision of drugs and equipment, his time in embryo recovery, and his instruction in the techniques of embryo evaluation and manipulation.

Dr. J. Duman for his generous provision of the hemolymph used in this study as well as his interest and sharing of knowledge of freeze tolerant insects.

Drs. R. E. Pearson, D. E. Mullins, and J. A. Lineweaver for their support, suggestions, and guidance throughout this research and for their evaluation of this thesis.

Dr. J. M. White for his departmental support in the provision of facilities, and opportunity for this research as well as for his evaluation of this thesis.

Judy Bame for her technical assistance in embryo recovery and her patient instruction in the preparation of this, as well as other manuscripts.

June Mullins for her assistance in the photography and illustrations used throughout my research as well as for this manuscript.

Mr. Chuck Miller, Mr. Harold Nester and the farm crew for their assistance and cooperation.

My friends and family for their support and encouragement.

And last, but not least, the lovely ladies of the misfit and milking herds who gave of themselves so that this research might be conducted.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Chapter I

INTRODUCTION

The bovine embryo transfer industry has become well established in several countries including the United States. The economic as well as the genetic implications of embryo transfer are quite profound to the modern cattle breeder. Some of the advantages that embryo transfer affords the cattle breeder include; ability to increase a genetically superior cow's productivity by allowing her to produce many more calves over her lifetime than would have normally been possible, permit cows with certain physiological problems which prohibit a normal pregnancy to produce embryos which are transferable to surrogate mothers, provide AI organizations a means to quickly obtain multiple offspring from a particular mating and determine carriers of certain recessive traits, and finally, as a research tool, to increase our general knowledge of the bovine reproductive tract and early pregnancy, and thus hopefully permit an increase in reproductive efficiency. In addition, embryo transfer has permitted an international marketing system of embryos by cattle breeders and investors.

Modern embryo transfer however, is not without its disadvantages and one of the most important is the relatively

high cost involved with the production of each offspring by such technology. A major reason for this cost is the preparation and maintenance of donor and recipient cows. It is often recommended to have ten to fifteen recipient cows programmed to be in synchrony, (re. estrous cycle), with the donor cow in order to receive embryos obtained from flushing the donor cow. When several embryos are recovered from the donor cow this expense is justified, but when few or none are recovered, the cost incurred in the preparation and maintenance of recipients is a profitless investment. One method to eliminate recipient related costs employed by many of todays embryo transfer firms is the preservation of embryos at low temperature (-196 C) until the time a recipient becomes available. In effect, the embryos are readied for the recipient rather than vice versa. In addition to the reduction in recipient related costs, a further advantage of the cryopreservation of bovine embryos is that the donor and recipient cow need not be in close proximity, thus facilitating the previously mentioned international and national marketing of embryos.

Although many embryo transfer firms offer the freezing of embyros as one of their services, survival of frozen-thawed embryos is still considerably lower than that of freshly transferred embryos. The actual survival rate of

frozen vs unfrozen embryos is confounded because quality and viability of embryos subjected to and recovered from the freezing process differs markedly among the many reports in the literature. Thus, claims made by commercial embryo transfer units who offer the freezing of embryos as part of their services as well as results reported in the scientific literature, are difficult to compare. Nevertheless, current state of the art methods of freezing result in sufficient embryo loss to make routine use of frozen embryos impractical. The potential economic advantage of successful embryo freezing provides a strong stimulus for further research in this area.

The ability for living cells to withstand freezing is far from impractical. Freeze tolerant insects have the ability to withstand ice formation within their bodies. This freeze tolerance is due to various compounds contained within the hemolymph of the insect, some of which serve as antifreezes while others, called ice nucleating agents actually induce a protective extracellular freezing which results in cell dehydration rather than the formation of lethal intracellular ice. This is accomplished by mechanical seeding of ice in freezing embyos. Because both the insect and embryo are multicellular organisms, they appear to encounter similar problems during freezing. Therefore, the

application of the insect's natural system of freeze tolerance may prove promising to the survival of freeze-thawed bovine embryos.

The overall objective of the research reported in this thesis was to evaluate the effects of inclusion of hemolymph from a freeze tolerant insect in the freezing medium on post-thaw survival of bovine embryos as well as to investigate the importance of seeding temperature. The specific objectives were as follows: 1) to evaluate the effects of the temperature at which ice formation is induced in the medium, (-5 C vs -7 C) on the in vitro development of frozen-thawed bovine embryos, and 2) to determine the effect of the inclusion of a biological ice nucleating agent, (hemolymph) at a low level, (.1% v/v) and at a high level, (10% v/v) on the in vitro development of frozen-thawed bovine embryos.

Chapter II

LITERATURE CITED

HISTORY OF CRYOBIOLOGY LEADING TO FREEZING OF EMBRYOS

The relatively young science of cryobiology deals with the effects of below zero temperatures on living organisms. It has only been within the last 25 years that cryobiology was recognized as a distinct area of biological science. Experimentation with low temperature preservation of living cells however is not new. As cited by Polge (1980), Mantegazza in 1866 reported the survival of human spermatozoa frozen to -17 C and Jahnel noted in 1938 that human spermatozoa could survive after freezing and storage at temperatures of frozen CO_2 and liquid gases. In 1949 the important, albeit accidental discovery that glycerol provided protection to avian spermatozoa during freezing and storage at -79 C, was reported by Polge, Smith and Parkes (1949). Later, bovine spermatozoa were successfully frozen by Smith and Polge (1950) and shortly afterward the birth of calves following artificial insemination with semen which had been frozen and stored at -79 C was reported (Polge and Rowson, 1952).

The successful cryopreservation of spermatozoa led to experimentation with other cell types, many utilizing gly-

cerol as a cryoprotectant. One of these was the mammalian embryo. In 1953 Dr. Audrey Smith reported that 1% of 1 cell rabbit embryos continued to divide after freezing and thawing in a 15% glycerol solution. Experimentation with the cryopreservation of mammalian embryos over the subsequent 20 years provided much new information about low temperature biology. Successful freezing of embryos during this period was characterized by their ability to develop in vitro following freezing and thawing. Then in 1972, Whittingham and Wilmut in independent studies, reported the birth of live mice from frozen mouse embryos that had been stored at temperatures which, similar to semen, would allow long term storage of frozen embryos. (Whittingham, et al., 1972; Wilmut, 1972).

'Successful' freezing thus began to mean that not only high percentages of frozen-thawed embryos develop in culture, but also result in the birth of normal offspring when transferred to recipients. The economic as well as genetic importance of the cryopreservation of embryos of the large domestic species encouraged further experimentation in this area, and in 1973 Wilmut and Rowson reported the birth of a live calf from a frozen embryo. Thirty-three day 10 to 11 blastcysts were frozen at .2 C/min using 2.0 M dimethyl sulphoxide (DMSO) as a cryoprotectant. Thirty-one of the em-

bryos were recovered after thawing and 21 of these were transferred to 11 recipients. Seven of the 21 transferred were cultured for 24 h prior to transfer. Two hundred and eighty-six days following non surgical transfer of the embryos, a healthy bull calf was born and was believed to have developed from one of the cultured embryos (Wilmut and Rowson, 1973).

ATTEMPTS TO OPTIMIZE CRYOPRESERVATION OF EMBRYOS

Attempts to establish the optimum freeze-thaw survival for embryos from the large domestic species have led to experimentation with various media, cryoprotectants, cool and thaw rates, as well as with the cold tolerance of various embryonic stages. Procedures developed by Whittingham (1971) and Wilmut (1972) have been used for successful freezing of developmental stages ranging from recently ovulated, fertilized oocytes to hatched mouse blastocysts, as well as hatched sheep and goat blastocysts (Whittingham, 1974; Bilton and Moore, 1976; Willadsen, 1977). The flushing medium used by Whittingham in 1971 consisting of a modified Dulbecco's phosphate buffered saline solution containing sodium pyruvate, glucose, bovine serum albumin and antibiotic is still the one of choice today for collection, storage, transfer, and freezing of mammalian embryos.

Willadsen in 1977, using procedures based on those of Wilmut (1972), investigated the interaction of cooling rate and thaw rate for the freezing of 6 to 8 day sheep embryos. These freezing procedures involved the equilibration of the embryo to the final 1.5 M level of the cryoprotectant dimethyl sulphoxide (DMSO) in a stepwise manner followed by rapid cooling to -6 C and seeding. Various combinations of cooling rates and thawing rates were investigated. Following thawing, the DMSO was diluted out in 6 steps over a period of 30 min to 1 h at room temperature. With a freezing rate of 1 C/min. to -20 C, embryos survived only if rapid thawing (360 C/min) was used, whereas with a freezing rate of .3 C/min, survival was obtained with both rapid and slow thawing rates. Use of very slow cool rates, .1 C/min from -30 C to -60 C made slow thawing obligatory. These findings distinguished between 2 factors affecting embryo survival, one which operated when rapid freezing rates were used with slow thaw, and the other when slow freezing was followed by rapid thaw. Thus, by the selection of freeze and thaw rate, their respective damaging effects could be minimized.

Although these techniques formed the basis for freezing of many mammalian embryos, and very slow rates of cooling are still used, it has been demonstrated with bovine and mouse embryos that if slow cooling is terminated before -33

C and embryos plunged into liquid nitrogen, fast thaw rates can be used (Willadsen et al., 1977; Whittinghan et al., 1979; Whittingham, 1980). Although DMSO was used as the cryoprotectant in the majority of the early bovine embryo cryporeservation attempts, later reports indicated glycerol was at least as effective as DMSO for bovine embryos at the morula and early blastocyst stage (Bilton and Moore, 1979; Bouyssou and Chupin, 1982).

Trousen et al., in 1976 established that increased cold tolerance of bovine embryos coincided with compaction of morulae and blastulation. Further study of the effect of developmental stage on survival of frozen embryos by Mohr and Trousen in 1981, examined the ultra-structural changes associated with the freezing and thawing in bovine embryos at three developmental stages; day 5, day 7, and day 13. Day 5 embryos had many vesicles and primitive junctional regions between adjacent blastomeres and after cooling to 4 C, the distribution of organelles within the blastomeres as well as spacial arrangement of blastomeres was disrupted. Day 7 embryos were at early blastocyst stage and were characterized by a ring of trophoblast cells attached by junctional complexes enclosing a disc of embryonic cell. Although these complexes were structurally unaffected by freezing, damage invoked or apparent after freezing included loss of the in-

tegrity of the trophoblast leading to the collapse of the blastocoel. After collapsing few blastocysts were able to reform a ring of trophoblast cells. Day 13 embryos had 3 distinct cell types; a layer of trophectoderm, a disc of embryonic cells, and a continuous layer of endoderm cells lining the blastoceol cavity. After freezing and thawing the embryonic cells were structurally intact but trophoblast cells were substantially damaged. These observations led these researchers to suggest that damage incurred during freezing may be selective for one type of cell within the embryo and that the extent and nature of damage were dependent on developmental stage of the embryos. Morulae/early blastocysts and blastocysts might then be expected to respond to freezing and thawing in different manners (Mohr and Trouson,1981).

Experimentation with various supplements to the freezing medium have also been conducted. As recently as 1983 Bondioli et al., found no difference in the freeze thaw survival of bovine embryos frozen in media supplemented with bovine serum albumin (.5% W/V), or newborn fetal calf serum (20% V/V). The freezing media for both treatments consisted of Ham's F10 and 1.5 M glycerol. Following thawing, 31% of those frozen with bovine serum albumin developed in culture compared to 34% of those frozen with fetal calf serum. Dam-

age to the zona pellucida was high for both treatments groups, 35% for those frozen with serum albumin and 26% for those frozen with fetal calf serum (Bondioli et al., 1984).

Wilmut (1972) in his original freezing of mouse embryos, found that the cryoprotectant must be added to and removed from the intracellular and extracellular environment of the embryo in a stepwise manner to prevent osmotic shock and lysis of the cells. This requirement that the cryoprotectant be slowly removed from the embryos before transfer into the cow restricts the use of frozen embryos under field conditions because the embryo must be unloaded from the freezing container and manipulated through a series of cryoprotectant diluting solutions which requires both a microscope as well as skill in the handling of embryos. Because of these problems, one step dilution procedures as well as dilution directly within the freezing container have been examined. One step dilution utilizing sucrose to reduce the impact of the osmotic gradient during glycerol egress from the intracellular environment to the extracellular environment was explored by Chupin et al., (1984). Their aim was to evaluate the feasibiltiy of dilution of the cryprotectant by a sucrose solution within the straw followed by immediate transfer without any selection of the embryos. They used methods described by Renard et al., (1982) and a modifica-

tion of one described by Leibo in 1982. Embryos were frozen with 20% lamb serum and 10% glycerol in phosphate buffered saline. A solution of .25 M sucrose with 20% lamb serum was located in a fluid column adjacent to the one containing the embryo with the two separated by an air bubble. The freezing rate was .3 C to -30 C at which point the straws were plunged into liquid nitogen. A rapid warm water thaw was used and the straw shaken gently to cause merger of the columns. Overall pregnancy rate following transfer was 41.4%. Although there was not a stepwise deglycerolation control, these results compare favorably with reports in the literature for embryos survival where the cryoprotectant was removed in a stepwise manner (Renard et al., 1981; Tervit and Elsden, 1981).

The investigations of effect of freezing medium, cool rates, thaw rates, and effect of embryonic stage on survival of frozen thawed embryos have aided in the current state of the art procedures for the freezing of bovine embryos. Using current technology, pregnancy rates of 20 to 60% as a percentage of embryos frozen have been acheived and reported in the scientific literature as well as by commericial firms (Tervit and Elsden, 1981; Leibo, 1981; Wallace and Henschen, 1984). This large range in survival and pregnancy rates reported is due primarily to selection of embryos to be cul-

tured, frozen or transferred. In some reports only very high quality embryos are frozen or only embryos appearing normal after thaw are cultured and transferred. In some cases, only embryos which expand in culture following thawing, and are therefore obviously viable, are transferred. In contrast, other studies include results of culture or transfer of all embryos subjected to freezing. Regardless of the success rates claimed, there is significant wastage and loss of embryos between freezing, thawing, and birth of live young to warrent continued efforts to improve cryopreservation of embryos. In order to understand the cause of freeze-thaw damage to embryos it would be helpful to understand the changes to which all cells are exposed at subzero temperatures and the factors most important to their survival.

CRYOBIOLOGY OF LIVING CELLS

As the environmental temperature of a solution is lowered to below 0 C, (the freezing point of pure water), ice nucleation (formation) is initiated. Ice formation usually occurs at a temperature below the freezing point at which time the temperature quickly rises to the freezing point before continued cooling of the frozen solution ensues. The solution is considered supercooled when it is below the

freezing point without ice formation (Salt, 1965; Leibo, 1970). The original nucleation of ice may be spontaneous, (homogeneous) or induced, (heterogeneous). Homogeneous nucleation of pure water occurs at supercooled temperatures below -30 C; however, it is usually overidden by ice nucleation around a foreign substance at higher supercooled temperatures (Lusena, 1955; Ring, 1980). An ice nucleation point may also be introduced into a supercooled solution by a process known as seeding where a cold metallic object is held to the fluid column or an ice crystal is introduced into the solution. Once initial ice nucleation is begun, the pure water will freeze out of solution resulting in a concomitant increase in the solute concentration of the remaining liquid solution. As the temperature is lowered further, more ice forms, eventually reaching a point where nearly all the water is converted to ice. At a sufficiently low temperature specific for a given dissolved solute, the solute itself begins to crystalize out of solution. This temperature, the eutectic point, is the point at which the entire solution is converted from fluid to solid state (Leibo, 1976).

Cells or embryos to be frozen must contend with intracellular as well as extracellular fluids. Despite the fact that the freezing point of cytoplasm is usually above -1 C,

intracellular fluid often will supercool to -10 to -15 C even when ice forms in the extracellular medium (Mazur, 1970). This indicates that the cell membrane can, under certain conditions, delay the spread of ice crystal growth to the supercooled cell interior (Mazur, 1970). Once ice nucleation occurs in the extracellular fluid, the pure water surrounding the cell freezes out of solution leaving the embryo exposed to an increasingly hypertonic environment and causing the formation of an osmotic gradient across the cell membrane (Mazur,1970). Osmotic equilibrium is maintained by loss of intracellular fluid outward across the cell membrane, (dehydrating), or by the formation of intracellular ice. Which of the two occurs is governed by factors such as cooling rate, presence of cryoprotectant in the medium and three basic physical properties of the cell. These properties include the cell's surface to volume ratio, membrane permeability coefficient and temperature which relate change in membrane permeability to change in temperature (Mazur, 1970; Meryman, 1974; Saacke, 1980; Leibo 1981). All of these factors work in conjunction in determining the survival of the freezing cells since intracellular ice formation is lethal.

The bovine embryo has a relatively small surface to volume ratio, a low membrane permeability and a high tempera-

ture coefficient. The combination of these factors cause dehydration of the embryo during freezing in response to the osmotic gradient formed to be an increasingly slow and difficult process (Leibo, 1981). If an embryo is cooled too rapidly, the cells will not have enough time to dehydrate before reaching the intracellular freezing temperature and ice will form within the cell. Thus, the embryo is vulnerable to intracellular ice. Ice crystals formed during rapid cooling tend to be very small and numerous due to multiple points of nucleation. Small ice crystals have higher surface energies than large crystals and tend to shift and merge togther to reduce this surface energy at temperatures as low as -100 C (Mazur, 1970; Mazur, 1980). When this type of ice crystal growth occurs, a process known as recrystalization, these intracellular ice crystals function like many small knives and are believed to permanently disrupt membrane structure and thus result in lethal damage (Mazur, 1980).

On the other hand cooling too slowly can also be deletorious to embryonic survival. Very slow cooling rates subject a cell to an array of potentially lethal conditions known collectively as 'solution effects' (Mazur, 1970; Meryman, 1974; Whittingham, 1976). Three theories have evolved to explain how solution effects harm cells. The first, pro-

posed by Lovelock in 1953 suggests that cells are lethally damaged when high salt concentrations occured in the interior and exterior of the cell as it dehydrated, possibly altering the cell membrane lipids causing the membranes to lose their semipermeabilty. Meryman, in 1974 proposed the minimum cell volume theory which suggests that damage occurs not necessarily from the electrolyte buildup but that the cell cannot dehydrate to more than 55% of its original volume without permanent membrane structure damage. A third theory was proposed by Mazur in 1980 and suggests that lethal solution effect damage is expressed when the unfrozen extracellular fluids reach 8 to 12% of their original volume. This implies that it is the fraction of the external solution remaining unfrozen rather than the salt concentration per se in the unfrozen fluid which determine the damage. Although none of the above theories is accepted as in total, they all acknowledge solution effects due to the removal of water as a very real problem.

The addition of certain cryoprotectant compounds to the freezing medium has been found to partially alleviate solution effects damage; however as there is no one accepted theory as to how solution effects harm cells, there is no one explantion as to how these cryoprotectants afford their protection. Cryoprotectant compounds have been catagorized

as intracellular, depending on permeation of the cell membrane for effectiveness, or extracellular, those which do not require penetration in order to be effective. Glycerol and DMSO are both classified as penetrating compounds and are believed to function in a colligative manner both intracellularly and extracellularly while substances such as sugars, hydroxyethyl starch, and polyvinylpyrrolidone are considered non penetrating cryprotectants. Although the penetrating cryoprotectants are able to pass into most cells, their protection cannot be soley attributed to this permeabiltiy since Mazur and Miller (1974) demonstrated that cells rendered impermeable to glycerol still received some cryprotection. It has been proposed that the penetrating cryoprotectants act as a salt buffer to reduce electrolyte concentration both intracellularly and extracellularly (Leibo, 1976). Whatever the exact mode of action of these cryoprotectants, it is known that the solution in which they are contained will have less ice formation at a given temperature than that same solution wihout the cryoprotectant at that given temperature (Saacke, 1980). The non penetrating cryprotectants, in particular polyvinylpyrrolidone, are believed to coat the extracellular membrane. Therefore they act at the surface of the cell's membrane and are thought to stabilize the macromolecular structure of the membrane (Meryman, 1974).

It is obvious that the cooling and thawing rates of cells have great influence not only in determination of cell survival but also on the type of damage, (solution effects or intracellular ice) to which they may succumb. Using measured values for cell volume, membrane permeability constants for water, and the temperature dependence of permeability constants, Mazur (1970) was able to predict the volume of water in a cell as a function of temperature, and cooling rate, and thus determine the optimum cooling and thawing rates for a given cell type without the formation of intracellular ice. However, even using cryoprotectants and the predicted optimal cool rate, there is usually loss of some cell viability.

In freezing bovine semen, loss of 15 to 40% of the viable cells is not uncommon. This loss however is not of great practical importance because it is accounted for in number of sperm packaged for the insemination dose; therefore even with such losses there remains enough numbers of viable cells to maintain fertility. In the freezing of bovine embryos, loss of an as yet undetermined number of cells within the multicellular embryo can rapidly result in the loss of the entire embryo.

A possible explanation for cell loss due to freeze-thawing despite the use of the proposed optimum cooling

rates was suggested in work by Brower et al., in 1981. They theorized the existance of different environments to which a cell might be exposed due to the velocity of the ice front moving through the sample. The velocity of an ice front is greatest at the point of nucleation and lowest at the point most distant to nucleation. They tested this hypothesis by placing porcine spermatozoa in an extender containing 14% glycerol onto a microslide, the ends of which were sealed with clay to prevent evaporation. The slide was then placed in a thermoelectrically controlled cold stage under a microscope equipped with microphotographic and TV tape recording capabilities. The samples were cooled at 1 C/min until freezing commenced at which time the advancing ice front was thermally transformed from the normal dendritic growth or interface, to a plane front growth. This enabled them to observe the behavior of sperm in relation to the advancing ice front. They noted that the behavior of sperm varied with the velocity of the ice front. Boar sperm were pushed ahead of the interface where velocity was .2 μm/sec or less and at higher velocity of .7 μm/sec or more, the sperm were encapsulated by the ice. Therefore they proposed the existance of three distinct regions regarding cell survival. The first area would be in the immediate vicinity of initial ice formation. Here the ice front moved very rapidly due to

the very dilute solution. The next region would be characterized by a slowing of the ice front as more and more of the pure water was frozen out of solution thereby effectively increasing the salt or solute concentration. The last region would be characterized as a very hypertonic environment where the ice front moved slowly due to the very concentrated salt solution left behind when the water was removed as ice. Cells located in the first environment would be exposed to very rapid rates of cool and therefore be exposed to intracellular ice formation while cells located in the third region would most likely succumb to solution effect damage due to the very high solute concentrations. The highest rate of survival then would be expected in the intermediate region where the optimal cooling rate would most likely be expressed. However, they were not able to demonstrate this due to the fact that the bulk of the spermatazoa were forced to the bottom of the slide by a slanted ice interface. They reasoned that for an individual cell, the location of that cell in regard to velocity of the ice front was what determined the mechanism of cell damage.

They drew attention to the importance of the immediate environment of a cell at the time of freezing regarding survival and suggested that plane front freezing could result in harvesting cells from a region where conditions were op-

timum. However, of importance to the preservation of cells or tissues by freezing is the potential relationship of a cell to the site of ice formation or velocity or the advancing ice front.

NATURAL FREEZE TOLERANCE

Nature has developed its own strategies for the successful cooling of multicellular masses. Compatibility of freezing temperatures and cell survival is evidenced in certain species of insects which are termed as cold hardy. Cold hardy insects are generally catagorized as either being freeze tolerant (able to survive freezing of their body fluids), or freeze susceptible (able to resist freezing at low temperatures (Salt, 1966; Ring, 1980; Baust, 1981; Duman, 1982; Ring, 1982,).

Freeze susceptible species adapt by producing antifreeze agents which lower their freezing and(or) supercooling points. The location or protection of their overwintering site is also important. Antifreeze agents in freeze susceptible insects include low molecular weight polyols and sugars such as glycerol and sorbitol as well as high molecular weight thermal hysteresis peptides and proteins (Duman, 1982). The antifreezes such as glycerol and sorbitol depress the freezing point of water in a colligative manner,

with the freezing point and supercooling points decreasing as solute concentration increases. It has been found that the low molecular weight solutes such as the polyols and sugars lower the supercooling point of water approximately twice more than they lower the freezing point (Duman, 1982). In addition to this colligative effect of polyols and sugars on supercooling point it has also been proposed that they lower the supercooling point by masking the heterogeneous nucleation sites in freeze susceptible insects (Baust and Morrisy, 1975; Baust and Morrisy, 1977; Ring, 1980). The proteins and glycoproteins which result in a thermal hysteresis, were first noted for their antifreeze action in cold water marine fishes (Devries, 1980). These proteins cause a disparity between the freezing and melting points of aqueous solution of several degrees and are believed to function in this manner due to their molecular repeating structures which allow them to adsorb by hydrogen bonding to the surface of ice crystals at sites where ice crystal growth is favored (Duman, 1982). This masks the crystal and prevents its growth at the normal freezing-melting point temperature. In the beetle, Meracantha contracta, the thermal hystersis proteins were found to be stimulated by short photoperiods and thus these proteins are proliferated during the winter months. Because changing photoperiod as well as

temperature are required to stop production, the insect maintains freeze resistance during winter thaws (Duman, 1982).

Freeze tolerant insects are capable under certain conditions of surviving ice formation in their extracellular body fluid. These insects appear to contain many of the same antifreeze polyols and thermal hysteresis proteins and peptides as freeze resistant insects but supercooling is limited to a few degrees below zero (Van der Laak, 1982). This limitation of supercooling to less than 10 degrees below zero is due to the presence of extracellular ice nucleating agents which have the ability to organize water molecules into an ice like confiquration at a few degrees below the melting point (Zachariassen, 1982). These nucleating agents function to induce freezing of the extracellular fluid at fairly high subzero temperatures, resulting in the slow dehydration of the cells and thus minimizing the posibility of the formation of lethal intracellular ice. The presence of glycerol, polyols, and thermal hysteresis proteins alleviate solution effect damage once ice formation is initiated. Zacharriassen et al., (1982) found that efficiency of nucleation activity of the hemolymph from the beetle, Eleodes blanchardi was affected by varying nucleator concentration. The nucleating activity was only slightly

affected when diluted by a factor of up to 10^3 but decreased rapidly beyond this range. They proposed that if the presence of one nucleator molecule was sufficient to yield maximum nucleator activity, the supercooling points would be expected to remain invariably high until a dilution factor was reached which resulted in some samples being completely devoid of nucleators. These samples would be expected to have supercooling points identical to pure NaCl solution. They found that dilution by a factor of 10^4 caused marked decreases in supercooling points and but dilution up to 10^5 resulted in supercooling points that were equivalent to pure NaCl solutions. This gradual decrease in supercooling point with increasing dilution indicated that specific quanities of the nucleating agents were required for efficient nucleation activity. Zachariassen et al., (1982) also observed that with increasing dilution of hemolymph, there was an increase in the standard deviation of the supercooling points. They interpreted this to mean that with high nucleating concentrations, there was a lower degree of randomness in the nucleator process.

Nucleating agents are also found in freeze sensitive species but are located in the gut region while in the freeze tolerant species they are found in the hemolymph and it is the hemolymph nucleators which are believed reponsible

for the favorable extracellular freezing with avoidance of intracellular freezing (Zacharriassen and Hammel, 1976; Duman, 1982; Zacharriassen, 1982). The chemical composition of the nucleating agents in hemolymph are still largely uncharacterized. Denaturation of these nucleating agents by heating to 100 C suggests that they are proteinaceous in nature (Duman, 1982; Zachariassen, 1982). Duman and Patterson found that the nucleators of Vespula maculata queens were completely inactivated by bacterial protease treatment and had molecular weights in the range of 3500 Daltons (Duman and Patterson, 1978).

The application of some of these same cryprotectant components employed by the freeze tolerant insect seemed potentially favorable to the freezing of another multicellular mass, the bovine embryo. The hemolymph used in this study was obtained from the freeze tolerant larvae of the Crane Fly, Tipula trivittata (Say, 1870). It is known to have nucleation activity at dilutions up to 10^3 at around -6 to -8 C (J. Duman, personal communication.)

Chapter III

MATERIALS AND METHODS

SUPEROVULATION AND EMBRYO RECOVERY

Embryos used in this study were obtained from lactating and nonlactating Holsteins cows located at the Virginia Tech dairy farm. The nonlactating cows were maintained as a group on pasture year round which was supplemented with hay and corn stalks during the colder months. Lactating cows were housed in conventional free stall housing or in a stanchion barn and fed a complete mixed ration of corn or alfafa grass silage, high moisture corn, and protein supplement. Nonlactating cows were observed for estrus activity at least once daily. Lactating cows were observed for heat daily when turned out on a dirt exercise lot and also during movement to and from the milking parlor. Kmar heat detectors were used on all cows during superovulation. Before beginning the superovulation regimen, the reproductive organs of the cow were palpated to check for major anatomical abnormalities which could interfere with successful superovulation and embryo recovery. All cows used were observed for normal estrous cyclicity prior to superovulation. At least one normal heat was observed on cows before reinitiation of the superovulation regimen. Cows were placed on the supero-

vulation regimen on any one of days 9-14 post estrus (estrus considered as day 0). Eight, 6.25 mg doses of FSH[1] (1 ml) were administered at 12h intervals. Along with the seventh and eighth injection, 35 mg, and 15 mg, respectively, of prostaglandin, PGF2a[2] were administered to assure Cl regression. Upon first observation of standing heat following prostaglandin injection cows were artificially inseminated with frozen semen of good quality. At this time they were also administered 100 ug of gonadotropin releasing hormone.[3] Cows were inseminated again 12 and 24 h later.

Flushing procedures were based on those of Colorado State University (Elsden and Seidel, 1982) as modified by Lineweaver, (personal communication) and are outlined below.

Embryos were flushed nonsurgically from cows 6 to 7 days post-breeding and therefore were at the morula or early blastocyst stage of development. The flushing medium was based on that of Whittingham (1971) as modified by Lineweaver and consisted of modified Dulbecco's phosphate buffered saline (PBS) supplemented with heat treated fetal calf serum (HTFS) and antibiotic (see appendix, table 9).

---

[1] FSH-P Burns Bio-Tech

[2] Dinoprotromethamine, Lutalyse, Upjohn Co.

[3] Cystorelin, Abbott

At the time of embryo recovery cows were confined in a head catch and palpated to estimate number of CLs present on the ovaries. The tailhead was clipped, scrubbed with an iodine soap solution and sprayed with 70% isopropyl alcohol. The rectal and vulval area were cleansed of manure and an epidural[4] of approximately 5 ml was administered between the coccygeal vertebrae to reduce contractions of the rectum, vagina, and uterus during embryo recovery. A sterile 20-Fr, 5 cc balloon 2-way Foley[5] catheter was inserted through the cervix into the uterine body and the cuff inflated with 3 to 5 ml of flush medium. Care was taken to insure the cuff fit snuggly against the internal os of the cervix thereby allowing fluid to flow into both uterine horns. A sterile 20-Fr extension tube was then attached to the end of the Foley catheter by means of a sterile plastic connector in order to facilitate introduction and collection of fluid away from the vulva thus avoiding potential contamination. A total of 440 ml of flush medium was introduced into the uterus in 60 to 120 ml aliquots by means of 60 ml syringes attached to the extension. The uterine horns were gently massaged to work the 60 to 120 ml of fluid throughout the length of the horns after which the fluid was recovered by gravity flow

---

[4] Lidocaine, Butler

[5] C.R. Bard Inc., Urological division

back through the foley catheter and extension into a sterile collection bottle. Careful manipulation of the reproductive tract during recovery aided the expulsion of all the fluid. Recovered fluid was maintained at 37 C until it could be searched for embryos at which time the entire 440 ml was carefully poured into a series of sterile 100 ml beakers and allowed to sit for several min permitting the embryos to settle to the bottom of the beaker. The flushings containing the embryos were permitted to reach room temperature (20 to 25 C) at which all other reagents and media were maintained. Small aliquots of the flushings were poured off and searched using a stereomicroscope. As embryos were found, they were placed into a solution of fresh modified Dulbecco's PBS, supplemented with fetal calf serum and antibiotic (see appendix, table 9).

Embryos were graded for quality as follows; 1-excellent, 2-good, 3-fair, 4-poor and unfertilized. Only embryos rated 3 or better were used. Characteristics that were evaluated to establish embryo quality were: compactness of cells, symmetry of embryo shape, uniformity in blastomere size, uniformity of cytoplasmic density throughout all blastomeres, and the presence of extraneous material and(or) cell fragments (Shea, 1981; Elsden and Seidel, 1982). As seen in fig. 1.1, the unfertilized ova demons-

trated no evidence of cleavage with the center mass being dark, dense and compact. Often the center mass of the unfertilized ovum was 1-dimensional, or flattened when viewed from a side angle. A fair morula, which often would be considered transferable but not always freezable, was characterized as having an intact cell mass but was downgraded for the presence of extraneous cells and material, heterogenous cell density (blackened cells), and irregular shape or dispersed cell mass. Fig. 1.2 is an example of a fair morula which has an intact cell mass but lacks symmetry and has extraneous material and extruded cells outside the main cell mass. A good morula, as illustrated by fig. 1.3, was characterized as having a symmetrical cell mass with cells of fairly homogeneous density and a symmetrical zona pellucida. The good embryo would often deviate slightly from the excellent embryo by displaying some extrusion of blastomeres or material but yet exhibited a healthy cell mass. The excellent morula as seen in fig. 1.4, was symmetrical in shape, with an even, compact cell mass containing blastomeres of uniform size, shape, and density and was free from extraneous material. The zona pellucida was also intact an symmetrical in shape. Upon blastulation, distinction of embryo quality became much more subtle, however, basically the same criterea were used to evaluate the blastocyst. Fig. 2.1

through 2.4 are are examples of various stages of blastocysts ranging from an early blastocyst (fig. 2.1) in which the blastocoel cavity is just forming, to an extremely expanded blastocysts starting to hatch (fig. 2.4). The ideal blastocyst was symmetrical in shape and consisted of blastomeres of similar size and density lining a symmetrical, fluid filled blastocoel cavity which was free from any extraneous material. The inner cell mass was flattened to one side and again contained cells of a uniform density, shape, and size. After recovery and evaluation, embryos were randomly assigned to treatment groups with all groups receiving morula and blastocysts equal in quality and number.

FIGURE 1. DIFFERENTIAL INTERFERENCE CONTRAST MICROGRAPHS OF BOVINE OVA AND MORULAE (FIGURES 1.1 - 1.4) X 770

Fig. 1.1 Unfertilized bovine ovum. There is no evidence of cleavage and the center mass is relatively dark and compact. Unfertilized ovum often are also unsymmetrical and when viewed from the side, appear flat or one dimensional.

Fig. 1.2 A fair morula (rated 3). This embryo would be considered transferable but not a good candidate for freezing. The center cell mass is intact and compact but there is a lack of symmetry to the cell mass shape and lack of uniformity of blastomere size. Also present are cell fragments and extruding blastomeres.

Fig. 1.3 This is a good morula (rated 2). The main cell mass is intact, compact, and both the cell mass and zona pellucida are symmetrical in shape. The blastomeres are are of uniform size and density throughout. There are some extruded blastomeres and extraneous material between the cell mass and zona pellucida but not enough to interfere with development.

Fig. 1.4 This an example of an excellent morula (rated 1). The cell mass is intact and symmetrical in shape, with and even, compact cell mass containing blastomeres of uniform size and density. The zona pellucida is also symmetrical in shape.

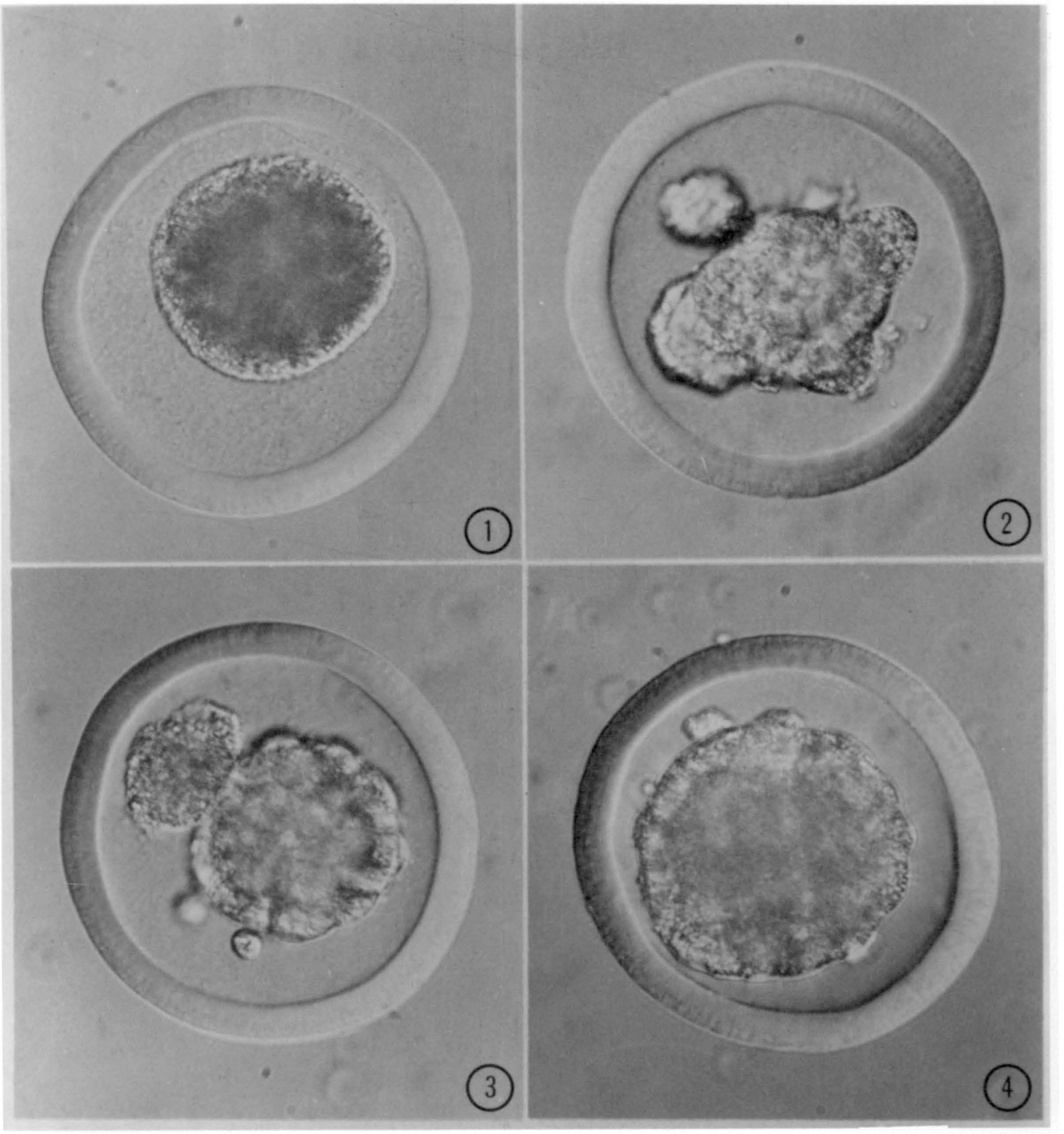

1
2
3
4

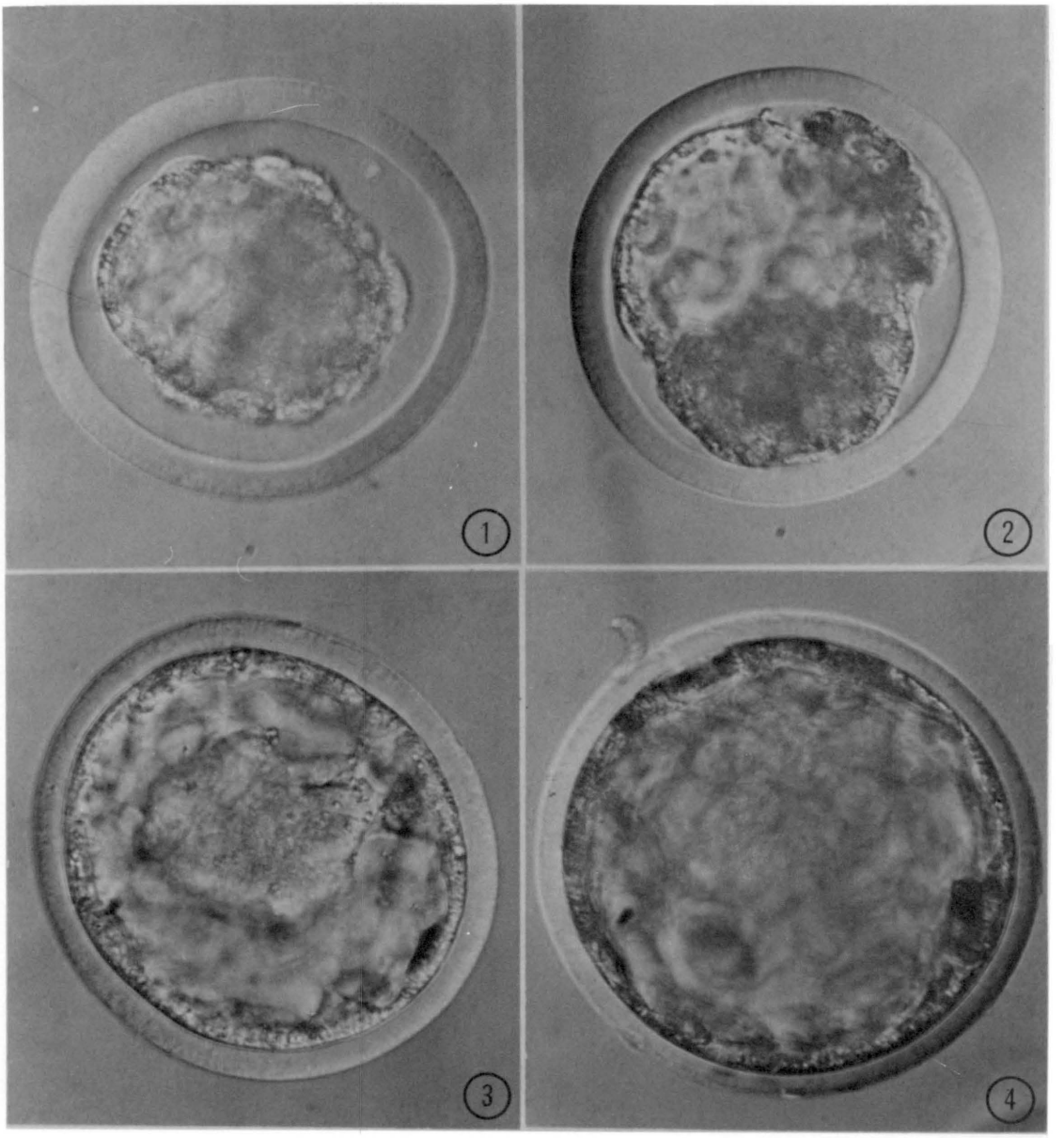
FIGURE 2. DIFFERENTIAL INTERFERENCE CONTRAST MICROGRAPHS OF BOVINE BLASTOCYSTS (FIGURE 2.1 - 2.4)
X 770

Fig. 2.1 An early but excellent day 7 blastocyst. Blastomeres are compact and beginning to migrate to one side to form an inner cell mass. The trophoblast cells are being pushed outward toward the zona pellucida by the expanding, fluid-filled blastocoel cavity.

Fig. 2.2 A good blastocyst. The inner cell mass in compressed to one side by the expanding blastocoel cavity which is extending outward toward the zona pellucida. There is some extraneous material which has been pushed outside the expanding ring of trophoblast cells but is not significant enough to interfere with development.

Fig. 2.3 An expanding blastocyst. Trophoblast cells line the inside of the zona pellucida surrounding the fluid-filled blastocoel cavity. The inner cell mass has been flattened to the top of this embryo and there has been a definite size increase resulting in the zona being stretched thin.

Fig. 2.4 Extremely expanded blastocyst just prior to hatching. Size increase is very obvious with the zona pellucida stretched very thin and beginning to tear. Trophoblast cells are flattened against the inside of the zona by the turgid, fluid-filled blastocoel cavity.


1
2
3
4

FREEZING PROCEDURE

Embryos were glycerolated in a stepwise manner by placing them in freezing medium containing 3.3% and 6.7% glycerol (v/v) for 10 min each followed by the final freezing medium containing 10% glycerol where they remained for 30 min before freezing. The final freezing medium was modified Dulbecco's PBS, fetal calf serum and 10% glycerol (Whittingham,1978; Elsden and Seidel, 1982) (see appendix, table 10). The various solutions were made up in sterile syringes fitted with .2 µ filters.[6] In treatments utilizing hemolymph, the concentration of hemolymph used was included only in the final freezing medium.

Embryos were frozen in .25 ml French straws,[7] one embryo per straw. Each embryo was drawn into the straw by means of a syringe fitted with a 16 gauge needle inserted into the factory seal end of the straw. As can be seen in fig. 3 within the straw each embryo was located in a column of medium with a column of air on either side. Polyvinyl alcohol powder was used to seal the straw (lab seal). The straws were soaked in ambient temperature water, 20-25 C, for at least 20 min to ensure that the plugs would be wetted and solidified. Each embryo was coded by a piece of tape placed

---

[6] Gelman Filtration Systems

[7] IMV, L'Aigle France

on the factory seal end of each straw on which was recorded an identification number corresponding to the quality, stage, and treatment of the embryo within.

A CRF-4E Programmable Biological Freezer[8] (methanol bath type) was used for freezing. Straws were frozen vertically in a rack with factory seal out of the methanol alcohol bath. The cool rate for all treatments was 1 C/min from ambient to -7 C, .3 C/min from -7 C to -35 C, and .1 C/min from -35 C to -38 C at which point the straws were quickly removed from the freezing chamber, wiped of excess methanol to prevent freezing together, and placed in goblets containing liquid nitrogen where they were stored (Elsden and Seidel, 1982). This was done as quickly as possible in order to minimize warming of the straw. All embryos were submerged for at least 2 wk before thawing and incubation.

CULTURE AND EVALUATION

Straws were thawed in 37 C water for approximately 30 s. As a precaution against the straw exploding, the factory seal was cut off immediately after removal from the tank and the straw was quickly placed in the thaw bath with the cut end out of the water. After thawing, the straw was wiped of

---

[8] FTS Systems

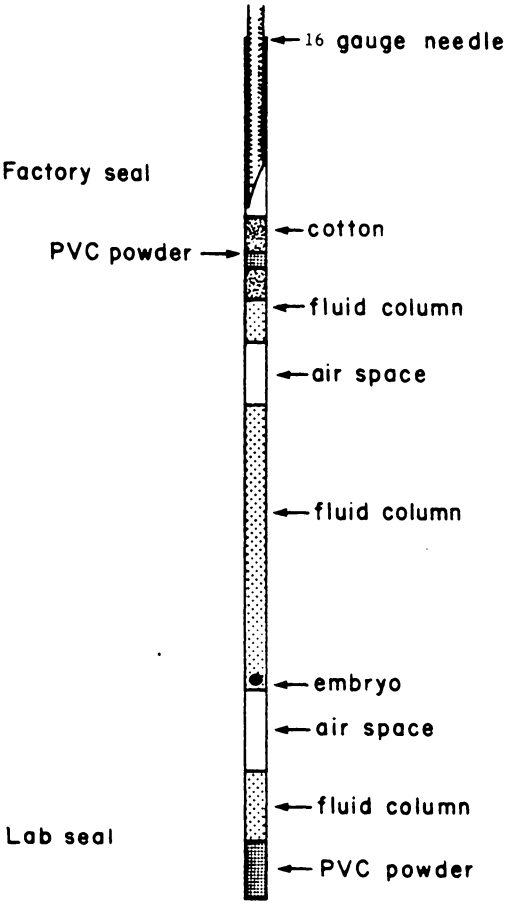


FIGURE 3. EMBRYO IN .25 ML FRENCH STRAW READY FOR FREEZING

excess water and examined under a stereomicroscope in order to determine the postion of the embryo. The straw content was drained into a watch glass containing fresh freezing medium by cutting the lab seal end and allowing the fluid to flow out by gravity. A syringe fitted with a 16 gauge needle was inserted into the end of the straw and fluid flushed in and out in order to remove embryos which might have adhered to the wall of the straw. The embryo was located in the dish, evaluated, and placed by means of a sterile glass Pasteur pipette into fresh freezing medium in the first well of a sterile multi-well tissue culture plate.[9] Embryos were deglycerolated in a stepwise fashion through freezing medium containing 10%, 8.3%, 6.7%, 5.0%, 3.3%, 1.7%, and 0% glycerol (v/v), each medium in a different well of the culture plate (Willadsen, 1978; Elsden and Seidel, 1982). Embryos remained in each solution for 10 min until they reached the final solution (0% glycerol), in which they remained for 30 min. After 30 min, embryos were again evaluated and each was removed from the final well and washed in fresh culture medium consisting of Ham's F10[10] supplemented with FCS, and antibiotic-antimicotic (Wright and Bondioli, 1981; Wright, personal communication) (see appendix, table 9). They were

---

[9] Lux Lab Tex, Division of Miles Laboratory

[10] Gibco

placed in a well of the tissue culture plate which contained several drops of culture medium and topped with a drop of parafin oil to prevent evaporation of the media. The paraffin oil had been equilibrated to the medium in the following manner. A 100 ml bottle of paraffin oil[11] from which 10 ml had been removed and replaced with 10 ml of .2 μ filtered Ham's F10 was shaken vigorously to mix the oil and Ham's F10 and a mixture of 90% N_2, 5% CO_2, 5% O_2 gas was bubbled into the bottle. The bottle was sealed and stored in a cabinet at ambient temperatures until needed. The medium and oil were re-equilibrated to the gaseous environment in the incubation chamber by placing them in the chamber for a few hours before introduction of the embryos. Several fresh, unfrozen embryos were cultured in preliminary testing to ensure that the system was functioning correctly.

In order to assess embryo survival, embryos were assigned a numerical value corresponding to the developmental stage reached in culture following thawing based on a modification of the system of Allen et al., (1981). As both blastocyst and late morula/early blastocysts stages were used in this study, two different systems were used for coding embryo development beyond their initial stage. The systems are shown in tables 1 and and 2 . Evaluation of embryo

---

[11] 125/135 Saybolt viscosity, Fisher

survival and developmental stage was made every 6 h for the first 24 h and every 12 h thereafter until no further development was observed and degeneration of the embryo was evident.

Least squares analysis of variance was used to determine the effect of treatment and initial quality on final developmental score. The model was:

$$Y_{ijk} = \mu + T_i + IQ_j + E_{ijk} \qquad (I)$$

where Y_{ijk} was the effect on mean final developmental score for the k^{th} embryo receiving the i^{th} treatment, and of the j^{th} initial quality where; μ was the mean final score; T_i was the effect of the i^{th} treatment; IQ_j was the effect of the j^{th} initial quality; and E_{ijk} was the random effect of the i^{th} treatment, and the j^{th} initial quality on the k^{th} embryo. In addition, mean time to reach each developmental stage in vitro was evaluated. The models were;

$$B_{ijk} = U + T_i + IQ_j + E_{ijk} \qquad (II)$$

$$EB_{ijk} = U + T_i + IQ_j + E_{ijk} \qquad (III)$$

$$HB_{ijk} = U + T_i + IQ_j + E_{ijk} \qquad (IV)$$

where B_{ijk}, EB_{ijk}, HB_{ijk}, were the effect on mean time (h), to reach blastocyst, expanded blastocyst, and hatching blastocyst stage for the k^{th} embryo, receiving the i^{th} treatment, and of the j^{th} initial quality, where; μ was mean time

TABLE 1. CODING SYSTEM FOR EMBRYONIC DEVELOPMENT OF MORULAE/EARLY BLASTOCYSTS

Code	Developmental stage reached in culture following thawing
0	Morula
1	Blastocyst
2	Expanding Blastocyst
3	Hatching Blastocyst
4	Hatched Blastocyst

TABLE 2. CODING SYSTEM FOR EMBRYONIC DEVELOPMENT OF BLASTOCYSTS

Code	Developmental stage reached in culture following thawing
0	Blastocyst
1	Expanding Blastocyst
2	Hatching Blastocyst
3	Hatched Blastocyst

to reach each stage; T_i was effect of i^{th} treatment; IQ_j was effect of the j^{th} initial quality; and E_{ijk} was the random effect of i^{th} treatment, and j^{th} initial quality on the k^{th} embryo.

HEMOLYMPH

The hemolymph used in this study was provided by Dr. J. Duman of the Biology Department, Notre Dame University, South Bend, Indiana. It was collected from crane fly (Tipula trivittata) larvae during February of 1983. The hemolymph was obtained by puncturing the cuticle of the larvae with a 25 gauge needle and allowing the hemolymph to drip into a test tube which was chilled in ice. The hemolymph was dialyzed[12] against distilled water at 4 C for 2 d and following dialyzation, was freeze dried (J. Duman, personal communication.)

The dialyzed, freeze-dried hemolymph was reconstituted to its original volume with phosphate buffered saline and was refrozen in .25 ml straws until needed. Straws were thawed in 37 C water immediately prior to the preparation of experimental media. Excess dilutions of hemolymph from a particular experiment were refrozen until needed again since reports indicated ice nucleation efficiency was not readily

[12] Arthur A. Thomas Cr. tubing-M.W. cutoff of 10,000 to 12,000)

affected by repeated freezing and thawing (Salt, 1965.)

In order to satisfy the stated objectives, 3 experiments were conducted. In Exp. 1, the effect of seed temperature (-5 C vs -7 C) and the inclusion of a low level, (.1%) of hemolymph on survival of frozen-thawed bovine embryos was investigated. In preliminary studies, hemolymph had been found to facilitate the ability to induce ice formation and growth at -5 C which was a very slow process without hemolymph, much different from ice nucleation and growth at -7 C. Moreover, spontaneous nucleation occurred at temperatures ranging from -5 C to -11 C in medium with concentrations of hemolymph up to 1 to 1000. Therefore the effect of the inclusion of a high level of hemolymph (10%) was examined in Exp. 2 and 3. Exp. 3 additionally sought to distinquish damage exerted by actual freezing.

Experiment 1

One hundred and fifty-two day 6 and 7 morulae/early blastocysts and blastocysts graded fair to excellent were randomly assigned to 1 of 3 treatments in order to evaluate the effect on embryo survival of temperature of seeding and the inclusion of hemolymph at a low level. The freezing medium for the control embryos (treatment 3) was a modified Dulbecco's PBS with 10% glycerol, while that for treatments

1 and 2 additionally contained .1% hemolymph v/v (see appendix, tables 10 and 11). Embyos were frozen as previously described. The control embryos were mechanically seeded when the cooling chamber temperature reached -7 C, by touching precooled forceps to the straw thus creating an ice crystal. Treatments 1 and 2 were similarly seeded at -5 C, and -7 C, respectively. Thus, comparison of treatment 2 and the control provided a means to evaluate the presence of hemolymph (seeded at -7 C). The effect of seeding temperatures possible by comparing hemolymph treatments only. Embyos were thawed, cultured and evaluated were as previouly described.

Experiment 2

Sixty-eight day 6 and 7 morulae/early blastocysts fair to excellent were assigned to 1 of 2 treatments in order to investigate the effect of a high level of hemolymph on survival of frozen thawed bovine embryos. Treatment 2 (without hemolymph) served as the control and was identical to the control in Exp. 1. The medium for treatment 1 was identical to the control medium but was supplemented with 10% hemolymph v/v (see appendix, tables 10 and 11). Both treatments were seeded at -7 C based on results of Exp. 1 which indicated no significant effect of seed temperature on post thaw

survival. Cooling, thawing, and culture were as previously described.

Experiment 3

Experiment 3 continued the investigation of the effect of a high level of hemolymph and in addition, sought to distinquish damage sustained due to freezing per se. One hundred and sixty two day 6 and 7 morulae/early blastocysts and blastocysts rated fair to excellent were randomly assigned to 1 of 3 treatments. Treatment 2 served as the control and was identical to the controls in Exps. 1 and 2. Treatment 1 was identical to treatment 1 in Exp. 2 with the freezing medium containing 10% hemolymph. Embryos in treatment 3 were handled identically with those in treatment 1 (containing hemolymph), except they were not subjected to freezing, i.e., they were glycerolated in the normal stepwise manner being exposed to a final medium with 10% hemolymph v/v, (the same as for treatment 1), in which they remained for approximately 35 min. Rather than cooling however, they were then deglycerolated in 6 stepwise dilutions in the usual post thaw procedure and placed into the culture system. Thus, comparison between treatment 1 and treatment 3 provided a basis to evaluate the effects of freezing per se. Survival assessment was as previously described.

Chapter IV

RESULTS

EXPERIMENT 1

Results for Exp. 1, comparing temperature at seeding (-5 C vs -7 C) and a low level of hemolymph (.1%) on freeze-thaw survival of embryos are presented in tables 3 and 4 for morulae/early blastocysts and blastocysts, respectively. Overall recovery of embryos was based on number of embryos cultured out of number frozen. The only reasons for not culturing a frozen embryo were that the straw exploded during thawing, or that the embryo was lost or had disintegrated beyond recognition. Overall, the embryos cultured represented 71% of those frozen and this value did not differ among treatments. Because two different developmental stages were frozen in Exp. 1, different scoring systems were used and separate analyses of variance were performed for each stage. It is apparent from tables 3 and 4 that morula/early blastocysts survived freeze-thawing better than blastocysts being 24.3% vs 2.9%, respectively. In fact, numbers of blastocysts frozen and surviving were so small that meaningful conclusions regarding effect of treatment or initial quality of the embryo on survival were not possible for this embryonic stage.

TABLE 3. EFFECT OF SEEDING TEMPERATURE AND A LOW LEVEL OF HEMOLYMPH (.1% V/V) IN THE FREEZING MEDIUM ON THE IN VITRO SURVIVAL OF FROZEN-THAWED BOVINE MORULAE/EARLY BLASTOCYSTS

	Seed -5 C with hemolymph	Seed -7 C with hemolymph	Seed -7 C without hemolymph
No. of embryos frozen	33	34	38
No. of embryos cultured[a]	22	24	28
Percent of embryos blastulating	27.3%	20.8%	25.0%
Mean time (h) to blastulation[b,c]	23.7 ± 7.6	16.6 ± 10.0	20.8 ± 7.4
Mean final developmental score[b,c]	.73 ± .23	.69 ± .24	.71 ± .22

[a]Differences between embryos cultured and frozen were due to embryos lost or disintegrating at thaw.

[b]Least squares means ± SE.

[c]Treatment differences within rows were not significant ($p > .05$).

TABLE 4. EFFECT OF SEEDING TEMPERATURE AND A LOW LEVEL OF HEMOLYMPH (.1% V/V) IN THE FREEZING MEDIUM ON THE IN VITRO SURVIVAL OF FROZEN-THAWED BOVINE BLASTOCYSTS

	Seed -5 C with hemolymph	Seed -7 C with hemolymph	Seed -7 C without hemolymph
No. of embryos frozen	18	15	14
No. of embryos cultured[a]	14	11	9
Percent of embryos expanding	14%	0	0
Mean final developmental score[b]	.14 $\pm$ .36	-	-

[a]Differences between embryos cultured and frozen were due to embryos lost or disintegrating at thaw.

[b]Mean $\pm$ STD differences within rows were not significant ($p > .05$).

For morulae/early blastocysts overall survival for treatments 1, 2, and 3, (the controls) was 27.3%, 20.8%, and 25.0%, respectively (table 3). Effect of seeding temperature was examined by comparing treatments 1 and 2, while effect of hemolymph was examined by comparing treatments 1 and 3. For either comparison, there was no significant effect of treatment based on mean final developmental score or on mean time to reach a particular developmental stage in vitro. There was also no significant effect of initial embryo quality or between initial embryo quality and treatment on embryo survival based on either mean final developmental score or time to reach a particular stage in culture for either morulae/early blastocysts or blastocysts. It should be pointed out however that of the 74 morulae/early blastocysts cultured 18 were excellent, 54 were good, and only 2 were fair.

For all three experiments, it was observed that immediately after thawing many embryos appeared morphologically normal, however, during deglycerolation they progressively degenerated. This was particularly true for blastocysts which frequently appeared normal at thawing but collapsed during deglycerolation. Damage to the zona pellucida by freezing was also quite common.

EXPERIMENT 2

The effect of a high, (10%) level of hemolymph on the survival of frozen-thawed morulae/early blastocysts is presented in table 5. There was no significant effect of treatment or initial embryo quality or interaction of the two, based on mean final developmental score or mean time to reach each developmental stage in vitro. It is apparent from table 5, numbers of embryos advancing 1 stage in culture, (blastulating) were approximately equal for both treatment. Embryos cultured after freezing represented 82% and 88% for treatments 1 and 2, respectively, and the disparity between embryos cultured and frozen was as stated in Exp. 1. A seeding temperature of -7 C was used because the results of Exp. 1 indicated no significant difference in survival based on seeding temperature.

The effects of a low level of hemolymph, .1%, (Exp. 1) and a high level, 10%, (Exp. 2), are compared in table 6. It is obvious that overall survival rate increased between the two experiments however, within each experiment there was no significant difference between a treatment and its control.

TABLE 5. EFFECT OF A HIGH LEVEL OF HEMOLYMPH (10% V/V) IN THE FREEZING MEDIUM ON THE IN VITRO SURVIVAL OF FROZEN-THAWED BOVINE MORULAE/EARLY BLASTOCYSTS SEEDED AT -7 C

	With 10% hemolymph	Without hemolymph
No. of embryos frozen	34	34
No. of embryos cultured[a]	28	30
Percent embryos blastulating	39%	40.0%
Mean time (h) to blastulate[b,c]	12.4 ± 2.5	11.1 ± 3.1
Mean final developmental score[b,c]	.77 ± .24	.66 ± .24

[a]Differences between embryos cultured and frozen were due to embryos lost or disintegrating at thaw.

[b]Least squares means ± SE.

[c]Treatment differences within rows were not significant ($p > .05$).

TABLE 6. COMPARISON OF RESULTS FROM EXPERIMENTS 1 AND EXPERIMENT 2 FOR IN VITRO SURVIVAL OF FROZEN-THAWED MORULAE/EARLY BLASTOCYSTS

	Experiment 1		Experiment 2	
	Seed -7 C with .1% hemolymph	Seed -7 C without hemolymph	Seed -7 C with 10% hemolymph	Seed -7 C without hemolymph
No. of embryos cultured after freezing	24	28	28	30
No. of embryos advancing one developmental stage in culture	5	7	11	12
Percent survival	20.8%	25.0%	39.3%	40.0%

EXPERIMENT 3

Results from Exp. 3 of this study expanded upon the investigation of the effect of a high level of hemolymph and additionally permitted examination of freeze damage per se by including a control that was glycerolated and deglycerolated but not frozen. Results are presented in tables 7 and 8. Once again as both morulae/early blastocysts and blastocysts were frozen, two separate analyses were performed. Again survival was so low among the blastocysts (table 8), that it was difficult to make meaningful conclusions about the treatment effect on this embryonic stage. Analysis of the morula/early blastocysts data, however, indicated a signficant, ($P>.0001$), effect of treatment as evidenced by least squares mean for final developmental score. Nonorthogonal contrasts were used to test for significant differences between final developmental scores of embryos frozen in the hemolymph and nonhemolymph treatments (1 and 2) and between embryos frozen in hemolymph and those cultured without freezing, treatments 1 and 3, respectively. There was no significant difference between treatments 1 and 2, but survival of embryos cultured without freezing was 30% better ($p>.0001$) than those frozen in hemolymph suggesting that the process of freezing itself was considerably detrimental to embryonic survival beyond the embryo manipulation pre and

post freezing. Initial quality of morulae/blastocysts had a significant effect on mean final developmental score with 56 excellent, 44 good, and 16 fair embryos cultured resulting in least squares means for final score of .78, .51, and .02, respectively. There was no significant difference of treatment or initial quality on mean time to advance one stage in culture. Embryos cultured represented 90.2% and 92.5% of those frozen in treatments 1, and 2, respectively (table 7).

TABLE 7. EFFECT OF FREEZING AND INCLUSION OF A HIGH LEVEL OF HEMOLYMPH (10% V/V) IN THE FREEZING MEDIUM ON THE IN VITRO SURVIVAL OF OF BOVINE MORULAE/EARLY BLASTOCYSTS SEEDED AT -7 C

	Frozen		Unfrozen
	with hemolymph	without hemolymph	
No. of embryos frozen	41	40	
No. of embryos cultured[a]	37	37	42
Percent of embryos blastulating	24.3%	32.4%	54.8%
Mean time (h) to blastulate[b]	19.7 ± 5.9[c]	25.4 ± 5.2[c]	25.3 ± 3.7[c]
Mean final developmental score[b]	.11 ± .14[c]	.27 ± .14[c]	.93 ± .12[d]

[a]Differences between embryos cultured and frozen were due to embryos lost or disintegrating at thaw.

[b]Least squares means ± SE.

[c,d]Least squares means with different subscripts within rows differed at $p > .0001$.

TABLE 8. EFFECT OF FREEZING AND INCLUSION OF A HIGH LEVEL OF HEMOLYMPH (10% V/V) IN THE FREEZING MEDIUM ON THE IN VITRO SURVIVAL OF BOVINE BLASTOCYSTS SEEDED AT -7 C

	Frozen		Unfrozen
	With hemolymph	Without hemolymph	
No. of embryos frozen	12	12	
No. of embryos cultured[a]	12	9	15
Percent embryos expanding	8.3%	0	13.3%
Mean final developmental score[b]	.08 ± .29	-	.13 ± .35

[a]Differences between embryos cultured and frozen were due to to embryos lost or disintegrating at thaw.

[b]Means ± STD differences were not significant ($p>.05$).

Chapter V

DISCUSSION

Throughout this research and the interpretation of the data obtained, one main realization evolved; that for every answer that is provided by an experiment on the freezing of mammalian cells, particularly, the bovine embryo, two more questions or possibilties become apparent. A considerable amount of knowledge has been gained regarding the freezing of bovine embryos in the past 20 years, with many of the early procedures being developed empirically. There are many factors which interact to affect embryo survival during freezing and thawing such as; cool rate, thaw rate, cryoprotectant level and type, freezing medium, and storage temperature (Wilmut, 1972; Willadsen, 1977; Bilton and Moore, 1979; Tervit and Eldsden, 1980; Schneider and Maurer, 1983). Even slight deviations in protocol can totally change an experiment and its outcome. This makes comparisons of studies in the literature quite difficult. The present study was designed to focus on events occurring at initial ice nucleation as influenced by seeding temperature, and(or) addition of hemolymph, (biological nucleating agent), to the freezing medium. Other parameters important to freeze survival were held constant, therefore potential interaction of seeding

temperature or hemolymph with any of the other important factors mentioned above could not be addressed.

In this study, there was an obvious increase in overall survival of morulae/early blastocysts between Exp. 1 and Exp. 2 with survival rates of 24.3% and 40%, respectively. This included a 12% increase in survival of the identically treated control embryos between Exp. 1 (25%) and Exp. 2. (36.7%). This general upward trend in survival rates may be attributed to several factors including improvements in technique in the handling of embryos during freezing and thawing, cow effect, and season of the year effect. Embryos used in Exp. 1 were collected during the months of September through December from nonlactating cows which were maintained on pasture supplemented with hay. Embryos used in Exp. 2 were collected during January and February from lactating cows which were maintained on a complete balanced ration and housed either in a stanchion barn or a conventional free stall barn. Thus, the condition of the cows from which the embryos were obtained differed between the two experiments, with cows used during Exp. 2 being maintained on a much more closely regulated diet than those used during Exp. 1. This difference in cow condition, as well as weather conditions may have had some impact on the viability of the embryos obtained during the two experiments despite no appa-

rent morphological difference in embryo quality. It is commanly reported by commercial embryo transfer firms, as well as in the literature, that both weather conditions and condition of the cow, affect cow response to superovulation and the quality of embryos obtained (Hahn and Schneider, 1979; J. Lineweaver, personal communication).

In both Exp. 1 and Exp. 3, overall survival of blastocysts (3.9%), was much less than morulae/early blastocysts (29.6%). Greater sensitivity of the blastocyst to freezing as compared to late morulae/early blastocysts observed in this study, is consistant with the literature. Several reports indicate that late morulae/early blastocysts tolerate freezing better than either earlier or later embryonic stages (Trouson et al., 1976; Willadsen, 1977; Willadsen, et al., 1978a; Willadsen, et al., 1978b; Bilton and Moore, 1979). Trouson et al., (1976) reported that precompaction stage, bovine morula were very susceptible to freeze-thaw damage. Willadsen et, al., (1978b) observed that very few 8 to 16 cell sheep embryos survived cooling to 0 C. One obvious reason for this would be a simple numbers game as there are fewer cells available for cleavage and development in these embryos than in later stage embryos, and the loss of cells due to freeze-thaw damage would have a greater impact on these early stage embryos. However, later stage blasto-

cysts, (day 9 to 13) have also been reported to be very vulnerable to freeze-thaw damage, in the bovine (Bilton and Moore, 1979) and the equine (Takeda et al., 1984). Bilton and Moore (1979) reported that of 7 and 9 day embryos frozen, only the day 7 embryos survived. Takeda et al., (1984), using freezing and in vitro culture methods identical to those used in this study, reported significant differences between numbers of blastocysts and morulae/early blastocysts developing in culture after freezing and thawing with survival rates of 21% and 62%, respectively.

In this study, it was observed that many embryos which appeared morphologically normal at thawing, degenerated during deglycerolation or simply failed to develop in culture. This was particularly true for the blastocyst which often was expanded at thawing but collapsed during deglycerolation. Similar observations were made by Bouyssou and Chupin (1981) who reported 79% of day 7 and 8 blastocysts appeared normal at thaw, but that only 32% of them had continued development in culture at 6 h. Mohr and Trouson (1981), using electron microscopy, reported damage to frozen-thawed embryos appeared to be cell specific with the embryonic disc cells of the blastocyst surviving freezing well, while the more differentiated trophoblastic cells displayed damage to mitochondrial, nuclear, and plasma membranes. Therefore,

many of the apparently morphologically "normal" embryos observed at thawing using light microscopy, may actually be permanently damaged at the ultrastructural level. As suggested by our observations, this damage is may possibly be accentuated by the deglycerolation process.

Many reports in the literature as well as procedures used by commercial firms, utilize a sucrose solution and a 1 or 2 step cryoprotectant dilution. Lehn-Jensen (1984) described a 1 step, in-straw dilution method using .5 to 1 M sucrose in a solution of phosphate buffered saline. Chupin et al.,(1984) reported pregnancy rates of 41.4% following non surgical transfer of embryos diluted of cryoprotectant within the straw using .5 M sucrose. The rationale behind dilution using sucrose solutions is that when frozen-thawed embryos are placed into a solution containing sucrose but not the cryoprotectant, the sucrose maintains sufficient osmotic pressure to prevent damage to cells during egress of intracellular cryoprotectant (Leibo and Mazur, 1978; Merry, et al., 1983). The use of stepwise deglycerolation in this study may have been less than optimal through exposure of the embryo to osmotic damage during deglycerolation which could have been reduced using a sucrose solution and a one step dilution method.

From this study, it is clear that the incorporation of hemolymph into the freezing medium at levels up to 10% v/v, did not improve survival of frozen-thawed embryos. Nevertheless, hemolymph did not appear to be toxic to the embryos. Hemolymph was included in the freezing medium in treatments 1 and 2 in the first experiment, each for a different purpose. In treatment 1, hemolymph facilitiated the induction of ice formation at -5 C which in preliminary studies had proven to be quite a difficult process without hemolymph, much different from induction of ice at -7 C (S. Whitman, unpublished data). Treatment 2 containing hemolymph was seeded at -7 C and was compared to a control (without hemolymph) also seeded at -7 C thus permitting evaluation of the effect of a low level of hemolymph on survival of frozen-thawed embryos. Exp. 1 demonstrated that the simple presence of a biological nucleating agent (i.e. hemolymph) in the freezing medium did not improve embryo survival at seeding temperatures of -7 C or -5 C. This lack of effect can be interpreted at the environmental level of the embryos as the embryos having had sufficient time to dehydrate when seeded at either one of these temperatures as initial ice crystalization and resultant dehydration is known to be an important phase in embryo survival. Initial ice induction in the medium surrounding the embryo at -5 C

apparently affords no advantage in allowing the resultant dehydration of the embryo to begin earlier, (in comparison to seeding at -7 C). The application of these results, could, however be limited by the complex interaction which exists between the seeding temperature, cool rate, as well as cryoprotectant type and concentration. The possibility exists that use of a more rapid cool rate or a higher seed temperature than those employed in this study may have resulted in a significant improvement in embryo survival in the presence of hemolymph.

Seeding temperatures reported in the literature range from -2 C to -7 C with post-thaw survival rates of 40% to 70% when followed by cooling at rates of or less than .3 C/ min (Willadsen et al., 1978b; Bilton and Moore, 1979; Tervit and Elsden, 1980; Schneider and Maurer, 1983). The effect of seeding temperature might become more apparent at higher cool rates, as the amount of time the embryo has in which to osmotically equilibrate could be more critical due to limitations of the embryo's low surface area to volume ratio, and low membrane water permeability coefficient. Since the initiation of ice formation in the freezing medium essentially changes the osmotic environment to which the embryo is exposed, it might be interesting to explore the initiation of dehydration of the embryo at above zero tempera-

tures through the use of compounds which would create an extracellular hypertonic environment in conjunction with a high subzero seeding temperature and a rapid cooling rate. As studies continue which have as their object to decrease the time involved in freezing embryos by allowing increases in cooling rate, then seeding temperature, or more precisely dehydration temperature, might prove to be a critical area for research.

Effect of seeding temperature, although not significant in this experiment may also require re-investigation if higher levels of hemolymph with ice nucleating agents, are included in the freezing medium i,e, levels above 10%. The nucleating agents present in hemolymph provide multiple sites of initial ice formation which in effect, changes the environment of cells with respect to the moving ice front. The number of nucleating points in a biological specimen would be related to the concentrations of the nucleating proteins or in our case, the hemolymph concentration. Thus, solutions with high concentrations of these agents may be expected to exhibit a more uniform velocity of ice front movement throughout the solution since there is less space between initial points of crystalization, while less concentrated solutions would display a more heterogeneous rate of ice movement. Velocity of ice crystal growth is greatest in-

itially and decreases with distance from the point of nucleation (Brower et al., 1980). The temperature of seeding also governs the velocity of ice growth. Allowing a solution to supercool appreciably before seeding will result in very rapid ice movement through the supercooled solution whereas seeding at a temperature nearer the freezing point will result in a much slower progression of the ice front and resultant more gradual dehydration of cells within the freezing medium. In the insect which contains high levels of these nucleating agents, the temperature at which initial ice formation occurs may be the primary method of controlling the velocity of the resultant ice growth. Thus if ice nucleating agents were included in freezing medium at high concentrations, a more obvious effect of seeding temperature may be apparent from a more uniform ice velocity exposed to each cell. Nevertheless, the experiments clearly indicate that the presence of a biological seeding agent per se is not sufficient and that higher concentrations should be evaluated before the concept is discarded.

In experiment 1, a .1% level of hemolymph was chosen to be included in the medium as this was the highest dilution at which we could still obtain spontaneous ice nucleation between -5 C and -11 C in our media which supercooled to approximately -14 C. This is in agreement with reports by Za-

chariassen (1982) and Duman (1982) who found at dilutions of 1 to 1,000, nucleation still occurred but beyond 1 to 10,000, the efficiency of ice nucleation was greatly decreased. The high level, (10%) in our study was based on preliminarly work with samples of fresh bovine spermatozoa which indicated that 10 to 50% v/v hemolymph was not toxic to these cells.

For either the low (Exp. 1) or high level (Exps. 2 and 3) of hemolymph, embryo survival was not improved based on mean final score or mean time to expansion for either morulae/early blastocysts or blastocysts. However, the hemolymph did not exert any obvious toxic effect per se as embryos in treatment 3 of Exp. 3 were exposed to the 10% hemolymph freezing medium, and glycerolation and deglycerolation but were not frozen, and their success rate in culture was significantly higher than those exposed to the same manipulations but frozen (treatment 1). The slightly lower survival of these embryos in culture as compared to fresh embryos, which have been reported to be as high as 70 to 90% by Renard et al., (1980) and Wright (personal, communication), was likely due to osmotic shock incurred during glycerolation and deglycerolation.

The apparent lack of toxcity with the levels of hemolymph used in this study indicate that investigation with

higher concentrations of nucleating agents may be warranted. In nature, accumulation of high levels of these nucleating agents, thermal hystersis proteins, and cryoprotectants during the winter months are among the strategies of the freeze tolerant for survival. It is known that freeze tolerant insects "acquire" their freeze tolerance by accumulating the antifreeze agents and nucleating agents during a process known as cold hardening (Baust, 1981; Van der Laak, 1982; Zachariassen, 1982). Moreover, as stated previously, the nucleator efficiency increases with increasing concentration of nucleating agent (Zachariassen, 1982). It is possible that high levels of hemolymph with nucleating agents and various cryoprotectants may afford increasing cryprotection to embryos. Possibly these compounds must be present at certain minimum levels which were not used in this study in order to exert their effect. Ring (1980), described the functions of cryprotectant compounds in the freeze tolerant insect as; increasing osmolality resulting in decreasing freezing point, acting as water binders thus decreasing the rate of ice crystal growth, decreasing dehydration within the cell, preventing decrease in cell volume below a critical size, stablizing membranes and enzymes, modifying ice structure, maintaining protein spacing, decreasing temperature at which recrystalization occurs during warming, and

enhancing multinucleation or initial ice formation at many sites simultaneously. This enhancement of multinucleation sites and modification of ice structure at high sub zero temperatures implies not only that there is a critical temperature of freezing, but that the mode of ice formation at the microenvironment level of the cell is critical to the survival of the insect.

In the previously mentioned work done by Brower et al., (1981), they suggested that the dendritic pattern of ice growth, which was likely to occur in most cryobiological systems, or a plane front growth (created experimentally), behave differently in regard to their effect on cell survival within the medium that is undergoing freezing. As suggested by Brower et al., (1981) three potential environments exist in a system that is undergoing freezing. The first environment is in the area of initial ice formation where cool rate is very rapid due to the low solute concentration or hypotonic environment, the next area is an intermediate zone where cool rate has slowed somewhat due to the slightly elevated solute concentration, and last, there is an area where the cool rate is very slow due to very high solute concentrations and a basically hypertonic environment. Cells located in the first area or a hypotonic environment where cool rate is very rapid, most likely succumb to intra-

cellular ice formation while those in the third area are exposed to very slow cool rates, a hypertonic environment, and succumb to solution effect stress. It is the intermediate environment, where cool rate provides the desired conditions and it may be that in the insect as well as in the freezing medium containing insect nucleating agents, that a high concentration of these nucleating agents create this sort of environment.

In viewing ice nucleation in general, multiple points of initial nucleation may allow for a more uniform ice growth velocity and therefore a more homogeneous osmotic environment by avoiding of areas of very low solute concentration (hypotonic environment) and rapid ice velocity, as well as areas of very high solute concentrations (hypertonic environment) where ice velocity is very slow. Thus incorporation of hemolymph (biological nucleating agents) from freeze tolerant insects into the freezing medium for embryos at levels higher than those used this study may result in a much more optimum osmotic environment.

In conclusion, there was no advantage to the survival of frozen-thawed bovine embryos of seeding at -5 C vs -7 C, or the inclusion in the freezing medium of Tipula trivittata hemolymph in concentrations up to 10% v/v. However, no toxic effects were apparent either. The potential of increased

cryprotection afforded by the inclusion of freeze tolerant insect hemolymph in freezing medium at higher concentrations and from other species remains to be investigated. The application of the strategies of freezing tolerant insects to the freezing of bovine embryos as well as other mammalian cells should be pursued.

LITERATURE CITED

Allen, R.L., K.R. Bondioli, and R.W. Wright. 1982. The ability of fetal calf serum, newborn calf serum and normal steer serum fo promote invito development of bovine morula. Therio. 18:185

Baust, J.G., and R.E. Morrisey. 1975. Supercooling phenomenon and water content in the over-wintering beetle, coleomegilla maculata. J. Insect Physiol. 21:1751.

Baust, J.G., and R.E. Morrisey. 1977. Strategies of low temperature adaptation. Proc. Int. Cong. Entomol. 15:173.

Baust, J.G. 1981. Biochemical correlates to cold hardening in insects. Cryobiol. 18:186.

Bilton, R.J., and N.W. Moore. 1976. In vitro culture, storage and transfer of goat embryos. Aust. J. Biol. Sci. 29:125.

Bilton, R.J. and N.W. Moore. 1979. Factors affecting the viability of frozen stored cattle embryos. Aust. J. Biol. Sci. 32:101.

Bondioli, K.R., C.B. Fruson, C.R. Looney, J.M. Massey, A.B. McGrath, P.C. Mertes, and A.J. Oden. 1984. In vitro survival of bovine embryos frozen in media supplemented with newborn calf serum or bovine serum albumin. Therio. 21:223.

Bouyssou, B. and D. Chupin. 1982. Two step freezing of cattle blastocysts with dimethylsulfoxide (DMSO) or glycerol. Therio. 17:159.

Brower, W.E., M.J. Freund, M.D. Bandindo and C. Ringwald. 1981. An hypothesis for survival of spermatazoa via encapsulation during plane front freezing. Cyrobiol. 18:277.

Chupin, D., B. Florin, and R. Procureur. 1984. Comparison of 2 methods for one step in straw thawing and direct transfer of cattle blastocysts. Therio. 21:455.

Devries, A.L. 1980. Biological antifreezes and survival in freezing environments. In: Animals and Environmental Fitness. pp. 583-607. Pergamon. Elmsford, New York.

Duman, J.G. 1982. Insect antifreezes and ice nucleating agents. Cryobiol. 19:613.

Duman, J.G., and D.L. Patterson. 1978. The role fo ice nucleators in the frost tolerance of overwinter queens of the bald face hornet. Comp. Biochem. Physiol. 59:69.

Eldsden, R.P. and G.E. Seidel Jr. 1982. Embryo transfer procedures for cattle. Animal Reproduction Laboratory, Colorado State University.

Hahn, J., and U. Scheider. 1979. Effect of the donor on egg quality and transfer results. Ann. Biol. anim. Bioch. Biophys. 19(5):1613.

Lehn-Jensen, H. 1984. Deep freezing of cattle embryos. Proc. 10th Int. Cong. on Anim. Reprod. and A. I. Champaign, Il. 4:2-1.

Leibo, S.P. 1976. Cryobiology. Yearbook of Science and Technology. McGraw-Hill. New York City.

Leibo, S.P. 1977. Fundamental cryobiology of mouse ova and embryos. In: The Freezing of Mammalian Embryos. Ciba Foundation Symposium. pp 69. Excepta. Medica. North Holland.

Leibo, S.P. and P. Mazur. 1978. Methods for the preservation of mammalian embryos by freezing. In: Methods in Mammalian Reproduction. pp 179-201. Academic Press Inc., New York.

Leibo, S.P. 1981. Preservation of ova and embryos by freezing. In: M.J. Bracket, G.E. Seidel, and S.M. Seidel (Ed.) New Technologies in Animal Breeding. pp 127-139. Academic Press Inc., New York.

Leibo, S.P. 1982. A one step method for direct nonsurgical transfer of frozen-thawed bovine embryos. In: International Congress: Embryo Transfer in Mammals. Annecy, France.

Lusena, C.V. 1955. Ice proprogation in systems of biological interest. III. Effect of solutes on nucleation and growth of ice crystals. Arch. Biochem. Biopys. 57:277.

Lovelock, J.E. 1953. The haemolysis of human red blood cells by freezing and thawing. Biochem. Biophys. Acta. 10:414.

Mazur, P. 1970. Cryobiology: The freezing of biological systems. Science. 168:939.

Mazur, P., and R.H. Miller. 1974. Survival of frozen thawed red cells as a function of the permeation of glycerol and sucrose. Cryobiol. 13:523.

Mazur, P. 1980. Fundamental aspects of the freezing of cells with emphasis on mammalian ova and embryos. Proc. 9th Int. Congr. Anim. Reprod. and A.I., Madrid. 1:99.

Merry, D.A., P.L. Allen, K. Krag, and R.W Wright. 1983. Sucrose dilution of frozen mouse embryos: Interaction of glycerol and sucrose concentrations. Therio. 20:325.

Meryman, H. 1974. Freezing injury and its prevention in living cells. Ann. Review of Biophysics. 3:341.

Mohr, L.R., and A.O. Trousen. 1981. Structural changes associated with freezing of bovine embryos. Biol. Reprod. 25:1009.

Polge, C., A.U. Smith, and A.S. Parkes. 1949. Revival of spermatazoa after vitrification and dehydration at low temperature. Nature. 164:666.

Polge, C., and L.E.A. Rowson. 1952. Fertilizing capacity of bull spermatozoa after freezing at -79 C. Nature. 169:626.

Polge, C. 1980. Freezing of spermatazoa. In: Low Temperature Preservation in Medicine and Biology. M.J. Ashwood-Smith and J. Farrant (Ed.) pp 45-64. Pittman Medical Limited, Kent.

Renard, J.P., J.P. Ozil, and Y. Heyman. 1981 Cervical transfer of deep frozen cattle embryos. Therio. 15:311.

Ring, R.A. 1980. Insects and their cells. In: M.J. Ashwood-Smith and J. Farrant (Ed.) Low Temperature Preservation in Medicine and Biology. pp 187-218. Pitman Medical Limited, Kent.

Ring, R.A. 1982. Freezing-tolerant insects with low supercooling points Comp. Biochem. Physiol. 173:605.

Saacke, R.G. 1980. What happens when a sperm is frozen and thawed. pp 6 Proc. 9th Tech. Conf. on A.I. and Reprod. N.A.A.B. Milwaukee. Milwaukee.

Salt, R.W., 1965. Factors influencing nucleation in supercooled insects. Can. J. of Zool. 44:117.

Say, T. 1870. American Entomology: A Description of the Insects of North America. John L. Le Conte M.D. (Ed.) Vol. II. pp 50. Estes and Lauriat, Boston.

Schneider, U., and R.R. Maurer. 1983. Factors affecting survival of frozen-thawed mouse embryos. Biol. Reprod. 29:121.

Shea, B.F. 1981. Evaluating the bovine embryo. Therio. 15:31.

Smith, A.U., and C. Polge. 1950. Survival of spermatozoa at low temperatures. Nature. 166:668.

Smith, A.U., 1953. In vito experiments with rabbit eggs. In: Mammalian Germ Cells. pp 217. Ciba Found. Symp. Churhill, London.

Takeda, T., and R.P. Elsden. 1982. Comparison of cryoprotectants for freezing embryos. Therio. 17:109.

Takeda, T., R.P. Elsden, and E.L. Squires. 1984. In vitro and in vivo development of frozen-thawed equine embryos. Proc. 10th Int. Cong. on Anim. Reprod. and A. I. Champaign, Il. 2:246.

Tervit, H. R., R.P. Elsden, and G. D. Farrand. 1981. Deep freezing 7 to 8 and 10 to 11 day old cattle blastocysts. Therio. 15:114.

Trousen, A.O., S.M. Willadsen, and L.E.A. Rowson. 1976. The influence of in vitro culture and cooling onf the survival and development of cow embryos. J. Reprod. Fert. 47:367.

Van der Laak, V., 1982. Physiological adaptations to low temperature in freezing tolerant PHYLLODECATA LATICOLLIS beetles. Comp. Biochem. Physiol. 73:613.

Wallace, R. A., and T. J Henschen. 1984. Future prospects for cattle frozen embryo banks. Proc. Ann. Conf. on A. I. and Embryo Transfer in Beef Cattle. Denver, Co. pp. 89.

Whittingham, D.G. 1971. Survival of mouse embryos after freezing and thawing. Nature. 233:125.

Whittingham, D.G., S.P. Leibo, and P. Mazur. 1972. Survival of mouse embryos frozen to -196 C and -296 C. Science. 178:411.

Whittingham, D.G. 1974. The viabiltity of frozen thawed mouse blastocysts. J. Reprod. Fert. 37:159.

Whittingham, D.G., M. Wood, J. Farrant, H. Lee, and J. A. Halsey. 1979. Survival of frozen mouse embryos after rapid thawing from -196 C. J. Reprod. Fert. 56:11.

Whittingham, D.G. 1980. Principles of embryo preservation. In: M.J. Ashwood-Smith and J. Farrant (Ed.) Low Temperature Preservation in Medicine and Biology. pp 65-84. Pitman Medical Limited, Kent.

Willadsen, S.M. 1977. Factors affecting the survival of sheep embryos during deep freezing and thawing. In: The Freezing of Mammalian Embryos. Ciba Foundation Symposium. pp 175-194. Elsevier. Excerta Medica. North Holland.

Willadsen, S.M., C. Polge, and L.E.A. Rowson. 1978a. The viability of deep frozen cow embryos. J. Reprod. Fert. 52:391.

Willadsen, S.M., C. Polge, L.E.A. Rowson, and R.W. Moore. 1978b. Deep freezing of sheep embryos. J. Reprod. Fert. 46:151.

Wilmut,I. 1972. The effect of cooling rate, warming rate, cryoprotectant agent and stage of development on survival of mouse embryos during freezing and thawing. Life Science. 11:1071.

Wilmut, I. and L.E.A. Rowson. 1973. Experiments ofn the low temperature preservation of cow embryos. The Vet. Rec. 92:68.

Wright, R.W. Jr. and K.R. Bondioli. 1981. Aspects of in vitro fertilization and embryo culture in domestic animals. J. Anim. Sci. 53:702.

Zachariassen, K.E., and H. T. Hammel. 1976. Nucleating agents in the haemolymph of insects tolerant to freezing. Nature. 262:35.

Zachariassen, K.E., J. G. Baust, and R.E. Lee. 1982. A method for quantitative determination of ice nucleating agents in insect hemolymph. Cryobiol. 19:180.

Zacharriassen, E.K. 1982. Nucleating agents in cold hardy insects. Comp. Biochem. Physiol. 73:4:557.

APPENDIX

TABLE 9. COMPOSITION OF MEDIAS FOR COLLECTION, HOLDING, AND CULTURE OF EMBRYOS

A.	Composition of Medium for Collection of Embryos	
	Dulbecco's Phosphate Buffered Saline (PBS) (Gibco)	500.0 ml
	Heat Treated Fetal Calf Serum (FCS) (Gibco)	5.5 ml
	Antibiotic-antimicotic (Gibco) with: 10,000 IU/ml penicillin, 25 mcg/ml Fungizone* 10,000 mcg/ml streptomycin	5.5 ml
B.	Composition of Holding Medium	
	Modified Dulbecco's Phosphate Buffered Saline (MPBS) with D-glucose at 1000mg/l and sodium pyruvate at 36 mg/l (Gibco)	13.5 ml
	FCS	1.5 ml
	Antibiotic-antimicotic	.15 ml
C.	Culture Medium	
	Nutrient Mixture F-10 (Ham's F-10) with L-glutamine (Gibco)	9.0 ml
	FCS	1.0 ml
	Antibiotic-antimicotic	.1 ml

TABLE 10. COMPOSITION OF STOCK SOLUTIONS FOR GLYCEROLATION, FREEZING, AND DEGLYCEROLATION

A. Stock Solutions

Solution A:	MPBS	40.0 ml
	FCS	10.0 ml
	Antibiotic-antimicotic	.5 ml
Solution B:	Solution A	18.0 ml
	Glycerol (Eastman Kodak)	2.0 ml

B. Glycerolation and Deglycerolation Solutions*

Stock Solution A	Stock Solution B	Approximate % Glycerol
2.0 ml	10.0 ml	8.3%
4.0 ml	8.0 ml	6.7%
6.0 ml	6.0 ml	5.0%
8.0 ml	4.0 ml	3.3%
10.0 ml	2.0 ml	1.7%
--	10.0 ml	10.0%

*Elsden and Seidel, 1982

TABLE 11. RECONSTITUTION OF DIALYZED, FREEZE-DRIED HEMOLYMPH, AND COMPOSITION OF HEMOLYMPH STOCK AND FREEZING SOLUTIONS

A. Reconstitution of Hemolymph

Hemolymph for Tipula trivittata (dialyzed and freeze dried)	6.0 ml*
PBS	to make 6 ml

*before freeze drying

B. Stock Solutions

Stock Solution C:	MPBS	8.5 ml
	FCS	7.2 ml
	Antibiotic-antimicotic	.34 ml
	Glycerol	4.0 ml
Stock Solution D:	MPBS	49.9 ml
	Reconstituted Hemolymph	.1 ml
Stock Solution E:	MPBS	.8 ml
	Reconstituted Hemolymph	.2 ml

C. Freezing Solution with .1% Hemolymph

Stock Solution C	5.0 ml
Stock Solution D	5.0 ml

D. Freezing Solution With 10% Hemolymph

Stock Solution C	1.0 ml
Stock Solution E	1.0 ml

The vita has been removed from the scanned document