30150 013496127

PRINCIPLES AND PRACTICE OF FREEZING BOVINE EMBRYOS.

MUHAMMAD NASIR MUSA

Dissertation in partial fulfilment for the degree of Masters of Science in Tropical Animal Production and Health.

University of Edinburgh 1982

ACKNOWLEDGEMENTS

I wish to express my sincere thanks to Dr. I. Wilmut, B.Sc., Ph.D. for his excellent help and encouragement and provision of materials used in this work.

The expert technical assistance of the staff of the Animal breeding and research organisation (Dryden) especially Miss Marjorie Thomson and Mr. Manson is gratefully acknowledged.

I am also grateful to Dr. Ruth Gatenby, Dr. A.J. Smith and Dr. D. Fielding for their help and encouragement throughout the course.

CONTENTS

		Page
	Abstract	(ii)
1	Introduction	1
2	Literature Review	3
2-1	Principles of Freezing	3
	(i) Introduction	3
	(ii) Response of cells to cooling	4
	(iii) Protective Compounds	5
	(iv) Mechanisms of Action	6
	(v) Response of Cells to Additives	9
	(vi) Addition temperature and exposure time	9
	(vii) Seeding	10
2-2	Sensitivity of some Embryos to Cooling in Ice	13
2-3	Early Methods of Freezing Mammalian Embryos	14
2-4	The Two Step Approach to Freezing Embryos	17
	(i) General Background	17
	(ii) Systems of Cooling	18
	(iii) Warming Rate	19
	(iv) Cryoprotective Agents	19
	(v) Methods of Dilution	20
	(vi) Integration of these Procedures	21
2-5	Assay	23
3	Materials and Methods	30
4	Results	35
5	Discussion	40
6	Conclusion	44
	Appendices	45
	References	50

ABSTRACT

This dissertation reviews the principles and practice of mammalian embryo preservation with particular reference to bovine embryos. The sections include principles of freezing, sensitivity of some embryos cooling, early methods of freezing mammalian embryos, four modern methods currently used, integration of various methods into practice and assay of survival.

A study has been carried out in an attempt to simplify the procedure of freezing and thawing. Embryos were recovered on day 7-8 from cross-bred heifers which had been induced to super ovulate by injection with PMSG. Some heifers also received an experimental injection of anti serum to PMSG. All the fertilized embryos which were recovered were frozen, regardless of their morphology. The embryos were allocated to four different treatment groups. In experiment I embryos were cooled in three different ways after being cooled at 0.3°C min. from -7°C to -33°C. They were either plunged directly into liquid nitrogen, held for 30 minutes, or cooled to -36°C at 0.1°C min. prior to plunging. Thawing was by agitating the test tubes containing the embryos in a water at 37°C until the ice disappeared.

Experiment 2 was a pilot trial to confirm whether embryos could tolerate direct transfer into and out of a medium containing 10% glycerol and either 0.25 or 0.5 M sucrose. In experiment 3, embryos were frozen and thawed in the presence of 10 per cent glycerol and either 0.25 or 0.5 M sucrose. After seeding at -7°C, embryos were cooled at 0.2°C to -15, -20, -25 or -30°C. After thawing, glycerol was removed by direct transfer of embryos into 1 ml of PBS.

In Experiment 4, embryos were cooled in the presence of 10% glycerol and either 0.25 or 0.5-M sucrose followed by direct plunging into liquid nitrogen. Glycerol was removed either by direct transfer of embryos into PBS or by transfer into a medium containing sucrose at the same concentration followed by transfer into PBS.

The survival rate of embryos cooled using three methods (Exp.I) was poor. Direct plunging at -33^{0}C gave the same survival rate as plunging at -36^{0}C after being cooled at 0.1^{0}C $min.^{-1}$ from -33^{0}C. By contrast none of the embryos survived when held for 30 minutes at -33^{0}C before plunging into liquid nitrogen. The result of the pilot trial confirmed that embryos can survive when transferred into and out of a medium containing 10% glycerol and either 0.25 or 0.5 M sucrose (85% survival). However, experiment 3 and 4 revealed that cooling embryos either at 0.2^{0}C $min.^{-1}$ to -15, -20, -25 and -30^{0}C or at 0.3^{0}C $min.^{-1}$ to -33 followed by plunging into liquid nitrogen is not compatible with survival irrespective of the way the glycerol was added and diluted.

It was concluded that holding embryos before plunging them into liquid nitrogen can be damaging. It was also suggested that the quality of embryos before freezing is critical for their future development after freezing and thawing. Although embryos frozen in the presence of sucrose did not survive, it was suggested that the cooling and warming procedures should be varied in search of a satisfactory method.

INTRODUCTION

The development of a reliable and simple method for long term preservation and resuscitation of embryos of the large domestic animals would greatly facilitate a more widespread use of embryo transplantation in the breeding of farm animals. Whereas low temperature preservation of semen and artificial insemination (A.1) have facilitated the extensive use of chosen bulls and use of progeny testing schemes in their selection, an effective method of exploiting the genetic potential of the female is still lacking. However, a full exploitation of the female genetic potential could be made with the development of an effective embryo freezing technique, by twinning, genetic control, non-surgical transfer and an International export of frozen embryos.

Generally, a very small proportion of cows produce twins after natural or multiple ovulation induced by gonadotrophin treatment. In these circumstances the most effective method of increasing the proportion of twin birth would be by transfer of an embryo to each uterine horn by a non-surgical procedure. However the application of this technique on a large scale depends upon the availability of a method for embryo preservation.

The potential values of genetic control would be considerable as they would enable breeders to select the most effective breeding procedure. However, this would be greatly facilitated by a long-term maintenance of a population without genetic change through a deep freeze preservation of embryos.

The principal method underlying successful embryo transplantation involves the synchronisation of oestrous cycles of the donors with those of recipients and the technique of super ovulation with

gonadotrophin to increase the number of embryos available. However, the response of the donors to gonadotrophin treatment is very unpredictable so the number of recipients is in most cases either too small or too large for the eggs available. As a powerful adjunct to such technique, embryo preservation can relieve the demand for having all the recipients at the correct stage of synchronisation for transfer on the day of embryo collection from the donor.

It has been shown that cattle could be transported easily as frozen embryos from one country to another (Bilton and Moore, 1977). However, the application of such technique on a commercial scale must await the development of a reliable and simple technique of freezing, thawing, and a non-surgical method of embryo transfer. In our search for the most simplified and efficient method of preserving embryos, we intend to confirm or otherwise the lethal effect of 30 minutes holding time at $-33^{o}C$ on bovine embryos during freezing prior to plunging into liquid nitrogen reported by Cockcroft (1981). Various combinations of cryoprotective compounds have been tried by many workers and the results were variable. It has been shown that bovine embryos could survive a combination of 10 per cent glycerol and either 0.25 or 0.5 Hl sucrose at room temperature (Wilmut unpublished). It is the aim of this paper also to find out the response of these embryos to such a combination of protectants at $-196^{o}C$.

2-1 Principles of Freezing

(i) Introduction

The main objective of freezing embryos is their maintenance in a state of suspended animation from which they may be resuscitated after a short or long storage period to continue normal development either *in vivo* or *in vitro*. The response of mammalian embryos to cooling is not unique since the same responses are reported for other cell types (Ferrant, 1980). During freezing and rewarming of a cellular system a large number of interrelated events take place an understanding of which is crucial for a successful preservation (Ferrant, 1977). These include:-

The formation of extra cellular ice
Increased extra cellular osmolarity
Increased extra cellular ionic strength
The formation of intra cellular ice
Increased intra cellular osmolarity
Increased intra cellular ionic strength
Osmotically driven water movements during freezing
Osmotically driven water movements during thawing
Increased concentration of cryoprotective agents outside the cells
Increased concentration of cryoprotective agents inside the cells
Solute transport across the cell membrane

It is generally believed that freezing injury is a consequence of two categories of physiological events. One associated with rapid cooling is the formation of intracellular ice crystals and their subsequent regrowth during thawing (recrystallization) (Nei, 1977).

The other associated with slow cooling, is a consequence of the change in the extra and intra cellular solution brought about by the freezing of solvent (water) into ice (Lovelock 1953). However, if cooling is slow enough that the intra cellular ice is not formed and yet rapid enough that the cells are not damaged by extensive exposure to high concentration of solute, they may survive the freezing process.

(ii) Response of cells to cooling

When a cell suspension is frozen *in vitro* freezing always begins in the solution bathing the cells (Ferrant, 1977). This is due to the heterogeneous nucleation from particulate matter in the solution. The ice can then spread throughout the extra cellular fluid, but it is halted by the barrier of the cell membrane (Ferrant, 1972). As a result of the freezing of the extracellular solute (water) there is a build up of osmolarity of the extracellular fluid, and the cells then begin to lose water and shrink.

Experience with freezing cells has shown that what happens subsequently to cells during freezing depends upon the rates of cooling and rewarming, the permeability of the cell membrane to water and the presence of cryoprotective additive in the medium. If the cooling rate is fast (faster than that giving optimal survival) the water inside the cell can not be sufficiently removed by shrinkage before a temperature is reached when the cell freezes internally giving rise to a large amount of intracellular ice (Ferrant 1980). In contrast, at a rate of cooling slower than that giving highest survival, the cells shrink (dehydration) in response to the increasing concentration of solute as water freezes out of the suspending medium during cooling.

Under these conditions, the cells are exposed to stress due to reduction in temperature (cold shock) and exposure to increasing concentration of extracellular solutes due to removal of extracellular solvent as ice (Ferrant 1980).

It has been shown that solute effect is one of the major causes of cell injury during slow cooling (Mazur 1970). Two hypothesis have been put forward to explain the mechanisms of the solute effect injury that accompanies slow cooling: 1, that it was due to a direct attack on the cell membrane by the increasing concentration of extracellular solvent (Lovelock 1953). 2, that it is due to the osmotic effect on the cell of a high concentration of solute (Merryman 1968). It was suggested that there is a progressive withdrawal of cell water until no more water can leave the cell due to the internal bulk of structures and solutes which cause a hydrostatic pressure gradient across the cell membrane.

(iii) Protective Compounds

It has been found that some compounds are able to protect cells from injury during slow cooling. The protective action of glycerol was first reported by Polge, Smith and Parkes (1949). Since then, compounds which have been shown to have cryoprotective properties range from Inorganic Salts (Trans and Bender 1960), to systemic alcohols (Polge and Soltys, 1959), Amines (Lovelock 1954) and some polymers such as PVP and dextran (Doebler and Richmond, 1962). Wilmut (1972) further classified them into three groups according to their osmotic effect. Dimethyl-sulphoxide (DMSO) was given as an example of a compound readily permeable to cell. Sucrose as a non-permeable but exerting significant osmotic effect, while a non-permeable polymer which has insignificant dehydrating effect (at the required concentration)

is exemplified by PVP.

Mazur (1970) suggests the following as desirable properties for cryoprotective compounds:-

1) Solubility at room temperature, as solubility confers the related property that once freezing begins in a system containing the compounds, the cryoprotectant will remain in solution.
2) A high capacity to form hydrogen bond and bind water.
3) Low toxicity to the cells they are supposed to protect.

(iv) Mechanisms of Action

The effect of these components is to lower the temperature at which a particular concentration of solute is formed and it is believed that the harmful effect of solute is less at lower temperatures. (Lovelock 1953). If a cryoprotective additive is present there is some depression of the melting point and less ice formation at any temperature during freezing. The degree of protection is a function of molar concentration of the protectant in the medium and of temperature (Mazur 1977). Fig. 1 and 2 illustrate the relationship between glycerol and DMSO concentration in the suspending medium and temperature, while Fig. 3 illustrates the relationship between salt concentration, temperature and concentration of cryoprotective compound (Glycerol). As the temperature decreases, there is a rise in the salt concentration but with increasing concentration of cryoprotectants there is a lower salt concentration at any given temperature. Thus cryoprotective agents lower the concentration of salt on cooling (Lovelock 1953). This property is shared by all

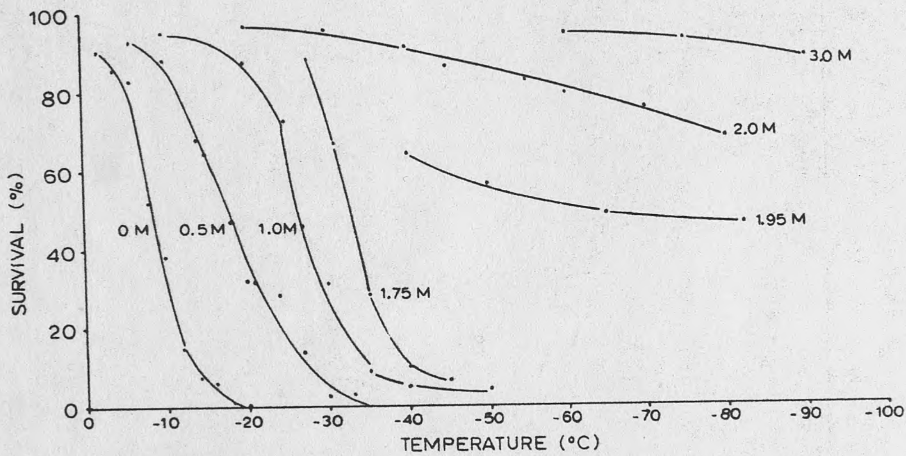


Fig. 1 Survival of frozen-thawed human red cells as a function of the concentration of glycerol in the medium (buffer saline) and as a function of temperature. (Mazur 1977).

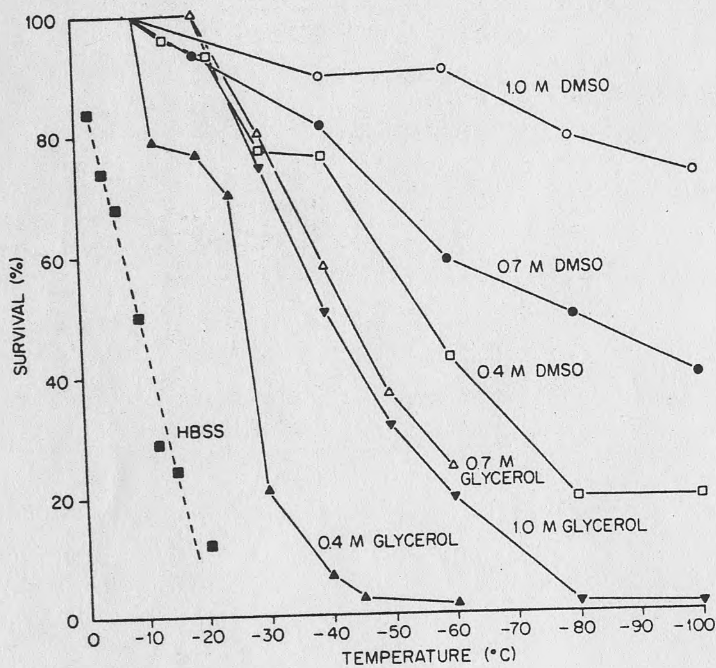


Fig. 2 Survival of frozen-thawed Chinese hamster tissue culture cells as a function of the concentration of glycerol or DMSO in the suspending medium and as a function of temperature. (Mazur, 1977).

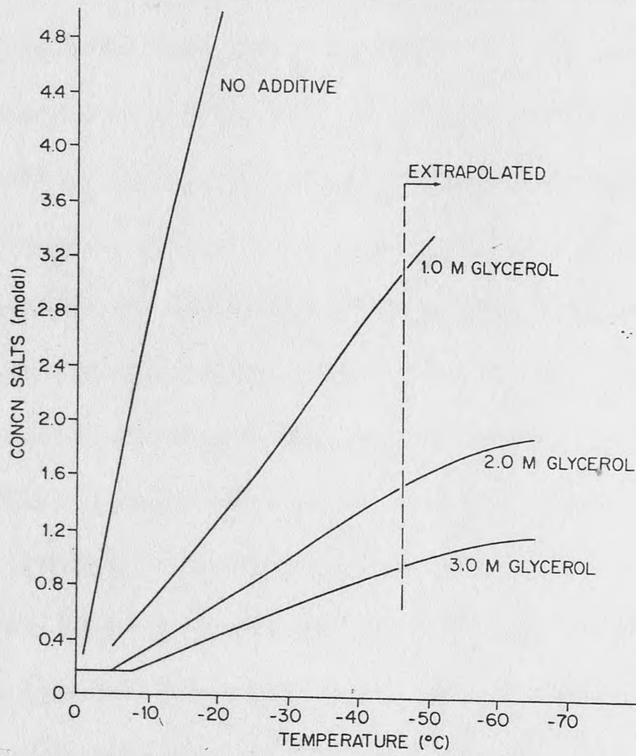


Fig. 3 Concentration of salt produced in the unfrozen solutions of glycerol in PBS Asa function of temperature (From Mazur, 1977).

groups of cryoprotectants whether they use permeable (DMSO, glycerol, ethylene glycerol) or non permeable (e.g. PVP, sucrose) (Merryman 1968).

(v) Response of cells to additives

The most common cryoprotective agent used (glycerol and DMSO) have cell membrane permeabilities which are much smaller than cell membrane water permeability (Ronald and Thomas, 1981). Consequently, in most instances large transient extracellular volume excursion should occur when cells are exposed suddenly to a high concentration of such protective agents. During introduction of permeable protectants, the cells initially shrink as water moves out of the cells due to the excess of osmotically active materials externally. The initial cryoprotective agent volume flow, however, should eventually be exactly balanced by the outward flow of water. This results in a transitory minimum cell volume. Subsequently, the osmotic gradient reverses its direction because the impermeable solutes are now more concentrated intracellularly than they are extracellularly and water enters the cells along with the permeable protectant. The volume changes finally come to an end when the difference in the extracellular and intracellular osmolarities of both the permeable and impermeable solutes vanish. During the removal of a permeable cryoprotectant from a completely equilibrated protected cell the cells behave in the opposite manner. That is, they first swell to a transitory maximum volume before shrinking to their final equilibrium.

(vi) Addition temperature and exposure time.

The permeation of cryoprotectants into the cell is a function of the initial solute concentration and temperature (Jakowski 1977).

The permeability to cell membrane increases with increase in temperature. Whittingham (1974) studied the effect of adding and diluting DMSO at various temperatures on the survival of frozen-thawed rabbit blastocysts. He found that blastocysts equilibrated with DMSO at 20°C and 37°C had a higher intracellular concentration of DMSO than those added at 0°C. This necessitated rapid removal of the cryoprotectants on thawing to minimize injurious osmotic effect. Tsunoda and Sugie (1977) showed that the proportion of live rabbits after transfer of frozen-thawed eggs was significantly higher when DMSO was added at 37°C than at 0°C. This further confirms the earlier observation of Whittingham and Wales (1974) on rabbit.

It was reported that an optimal survival rate of 8-cell mouse embryo was obtained after exposure to DMSO for 5 minutes at 0°C, and this survival rate increased when the exposure time increased to 30 minutes (Whittingham, 1980). The exposure time required for optimal survival to a given concentration of a protectant depends upon the stage of development of the embryo (Willadsen 1977). Late morulae and blastocysts were shown to require longer exposure time to DMSO at temperatures above 0°C. For sheep and cattle blastocysts, the total exposure time at room temperature to DMSO including stepwise addition is 40 minutes (Willadsen, 1977). Late morulae required longer exposure time because of the formation of tight junctional complex between the outer trophoblastic cells and this reduces the rate of permeation of the protectant into the intercellular spaces (Willadsen 1977).

(vii) <u>Seeding</u>

When media are cooled at the rate required for embryo preservation, nucleation rarely occurs at the freezing point of the

media and the samples may super cool as low as $-21^{\circ}C$ (Whittingham 1980). At ice formation, the latent heat of fusion causes the sample temperature to rise to that of the melting point of the medium while the bath temperature continues to fall at a constant rate (Whittingham 1980). The greater the degree of super cooling, the larger the temperature difference that will exist between cooling vessel and sample immediately after ice formation (Fig. 4). The increase in temperature during the phase change in itself is not harmful to the cells, but the subsequent increase in cooling rate detrimental, because super cooled samples cannot dehydrate until ice forms and subsequently the cells have insufficient time to dehydrate adequately before reaching the temperature where ice forms intracellularly (i.e. if they remain in the same cooling bath after ice induction). A variation in survival rates of 8 cell mouse embryos was reported when seeding took place at various subzero temperatures. The survival of these embryos cooled at $0.5^{\circ}C$ min. fell dramatically when ice was induced below $-7^{\circ}C$ and none survived super cooling to $-12^{\circ}C$ or below (Whittingham 1977). To avoid the deleterious effect of super cooling, samples are routinely transferred to a constant temperature seeding bath (after equilibrating with cryo-protectant) one or two degrees below the melting point of the medium and ice is induced to form. Whittingham (1980) described how this can be achieved by one of the following methods:-

1) Touching the surface of the suspending medium with either ice crystals in a pasture pipette or wire pre-cooled in liquid nitrogen.

2) Touching the outside of the ampules containing embryos with a dry ice or pre-cooled forceps or metal bar in liquid nitrogen.

3) Sharply tapping the ampules containing embryos.

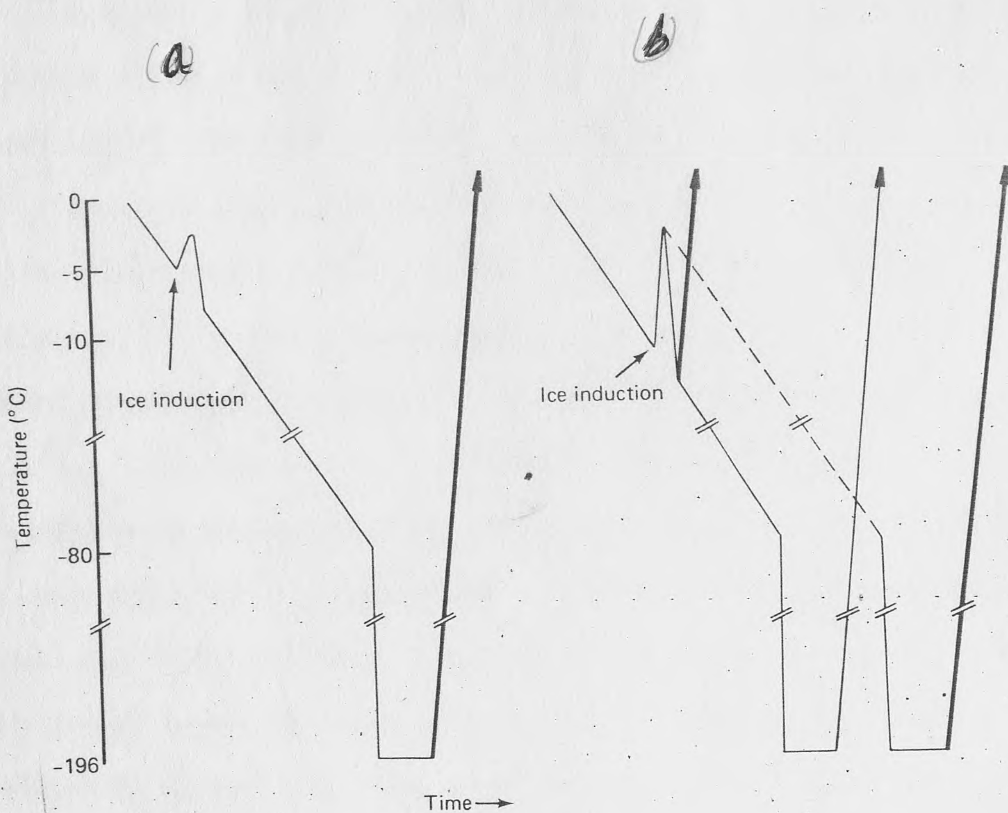


Fig. 4 Illustration of typical cooling curves showing the elevation of temperature resulting from ice induction at $-5^{0}C$ (a) and $-12^{0}C$ (b) and subsequent increases in cooling velocities as sample re-equilibrate with the temperature of the cooling bath. (From Whittingham, 1980).

2-2 <u>Sensitivity of some Embryos to cooling in ice.</u>

The first attempt to freeze embryos from farm animals revealed that some stages were killed by cooling in ice. Wilmut, Polge and Rowson (1975) examined the effect of cooling (rates) on bovine embryos. They found that early morulae were killed after a relatively rapid cooling (10°C min.) to 0°C, however, most of the late morulae survived cooling to 0°C and when frozen, thawed and transferred. The subsequent pregnancy rate equalled those of control morulae stored at 20°C. This suggests that different cleavage stages differ in their sensitivity to cooling to 0°C. Further evidence of the importance of the cleavage stage of development was reported by Trounson, Willadsen, Rowson and Newcomb (1976). They found that only 7 per cent of 8-24 cell bovine eggs survived cooling whereas 32 per cent of morulae (consisting of over 32 cells) developed to a normal blastocyst after thawing. The survival rate of morulae from this report is much lower than the one reported by Wilmut *et al* (1975) and they suggested that the morulae used by Wilmut and his co-workers were more mature. After conducting series of experiments, Trounson *et al* (1976) concluded that Day 5-6 cow embryos do not survive well after cooling and thus are not suitable for freezing. It was also reported that the resistance of sheep embryos to cooling increases with age and it was suggested that a change in susceptibility to damage by cooling apparently occurs during the period of 1-2 days that separates the morulae and blastocyst stages. (Willadsen, Trounson, Polge, Rowson and Newcomb 1976). The pre-blastocyst cow egg appears to be almost as susceptible to cooling damage as the pig (Wilmut 1972) and more susceptible than the sheep eggs.

2-3 Early Methods of Freezing Mammalian Embryos

Following the discovery of the protective action of glycerol to an avian sperm against the deleterious effect of cooling (Polge, Smith and Parke 1949), many attempts were made to preserve various tissues, organs and embryos of various animal species. In 1952, Audrey Smith (1952) showed that exposure of fertilized mammalian embryosto a low temperature (-70°C and -196°C) was not incompatible with further development. Although the survival rate was poor (1 per cent), Smith was optimistic for a better result with appropriate modification in the techniques. Similar poor survival rates have been reported in embryos of other laboratory animals (Whittingham 1971) and sheep (Averil and Rowson 1959). However, in 1972, the first successful preservation of mammalian embryos was reported by two independent groups of workers (Wilmut 1972, Whittingham, Leibo and Mazur, 1972). They achieved their results by a careful manipulation of the factors that might affect the survival of the frozen embryos namely:- cooling rate, thawing rate and appropriate choice of cryoprotectant. They reported that mouse embryos at all stages of development could survive slow freezing and slow thawing.

The survival rates of about 90 per cent were obtained when mouse embryos at 1-cell to early blastocyst were cooled at 0.2-0.8°C min. and warmed at 4-25°C min. Because embryos at these stages are contained within a zonapellucida, it was suggested that the presence of zona has a considerable influence on the response of mouse embryos to cooling (Wilmut 1972), but further research showed that this was not the case (Wilmut, personal communication).

The cryoprotectants used were 1.5M DMSO and 1.0M glycerol and both were found to be effective in protecting the embryos against

cooling injury. When media containing 2.0 M DMSO was added to embryos at 20°C, they shrank and then re-expanded after 5 minutes. Direct addition of medium containing 2.0 M DMSO was shown to have damaged the embryos irreversibly and that prolonged exposure of embryos to DMSO was also damaging (Wilmut 1972). However, the optional addition rate of DMSO appeared to be an increment of 0.5 M to 1.0 M at 5 to 10 minute intervals.

When eight-cell mouse embryos were exposed to 1.0 M DMSO at 0°C for various periods before freezing, it was found that survival was best at 1.6 minutes (Whittingham <u>et al</u> 1972). The survival of embryos cooled at 0.8°C min. after 17 minutes exposure depended on the concentration of DMSO and it was noted that a concentration of 1.0 to 1.25 M appeared to be the best (Whittingham <u>et al</u> 1972).

Although both methods used very slow cooling, the details of the cooling procedures for the two reports are quite different (Appendix I and II) and the findings are similar. Both emphasised the superiority of slow over rapid cooling rates. Although Wilmut (1972) obtained a good result when slow cooling (0.22°C min.$^{-1}$) was terminated at -70°C, with direct plunging into liquid nitrogen (-196°C), Whittingham <u>et al</u> (1972) observed that slow cooling (0.3°C min.$^{-1}$) to -110°C was better than plunging at -80°C. Slow warming (4-25°C min.$^{-1}$) which prevents injurious osmotic shock yielded far more survivors when cooling was between the rates of 0.3°C to 1.9°C min. It was also noted that warming at 4°C min.$^{-1}$ was better than at 25°C (Wilmut, 1972).

Routinely, the cryoprotective agents are added by a step-wise dilution method. The effectiveness of this method was shown by the attainment of high survival rates of about 84 per cent of unfrozen embryos suspended in 1.0 M DMSO at 0°C for up to 90 minutes.

(Whittingham et al 1972). This reduces the osmotic damage and toxic effect of this agent reported by Whittingham and Wales (1969).

The initial assumption was that embryos would only survive if cooled slowly and warmed slowly but subsequent research showed that the effect of rapid cooling depended upon the temperature range at which it occurred. If 8-cell mouse embryos were cooled slowly to $-30^{o}C$ before being cooled rapidly, none survived slow warming. By contrast, if slow cooling ($0.3^{o}C\ min.^{-1}$) continued to $-50^{o}C$ or $-60^{o}C$ before rapid cooling began the survival was very high. (Leibo 1977).

The application of the procedure of slow cooling and slow warming to early blastocyst of sheep and cattle was successful, but the survival rate was poor being around 30 to 50 per cent. (Willadsen et al 1976 , , Trouson et al, 1978). Contrary to the earlier expectations, it was also found that if the concentration of DMSO was high, or if later stages of embryos were being frozen, that the embryos tolerated rapid warming (Wilmut and Rowson 1973, Willadsen et al 1977, Trouson et al 1978). These observations led to a revolution in methods of freezing embryos.

2-4 The Two Step Approach to Freezing Embryos

(i) A major change in the methods of freezing embryos followed the demonstration that embryos which had been plunged into liquid nitrogen from temperatures around -35°C only survived if warmed rapidly. Sheep embryos in 1.5 M DMSO were cooled at 0.3°C to a temperature between -30°C and -54°C and plunged into liquid nitrogen. The warming rate was either 4°C $min.^{-1}$ (slow) or 360°C $min.^{-1}$ (rapid). If the warming rate was slow, survival was obtained only in embryos plunged from -54°C, whereas with rapid warming, survival was optimal when embryos were plunged from -42°C or -48°C (Willadsen *et al* 1978).

Similar observations were made with 8-cell mouse embryos cooled in 1.5 M DMSO at 0.3°C min. to a temperature between -30°C and -60°C before being plunged into liquid nitrogen (Whittingham *et al* 1972). If the warming was slow, embryos only survived if they had been cooled to -60°C at 0.3°C $min.^{-1}$ before being plunged into liquid nitrogen. By contrast, if the warming rate was rapid, embryo survival was high if the embryos had been plunged from a temperature between -30°C and -40°C.

It has been suggested that when slow cooling is continued to lower temperatures, dehydration continues and that at temperatures around -60°C there is insufficient water remaining inside the cell to form intracellular ice (Leibo 1977). These dehydrated embryos can be rewarmed slowly as recrystallization is no longer a problem. However, if such embryos are warmed rapidly, they are seriously damaged (Willadsen 1977). The damage to those embryos could result from changes which would occur with the sudden release of water from the melting ice. Also the relatively low permeability of these embryos to water could delay dehydration and prolonged exposure to high intra-cellular solute concentration at high temperature could result in

damage from solute effect (Whittingham 1980).

Since then, all methods of freezing and thawing cattle embryos have employed a high temperature for plunging. A great variety of different combinations of plunging temperatures, cooling rates, warming rates and different cryoprotective agents have been compared.

(ii) Systems of Cooling

Four different systems of cooling have been developed for freezing cattle embryos:-

1) In the Rapid Method described by Willadsen (1980) embryos are cooled at $0.3^{0}C$ $min.^{-1}$ to $-30^{0}C$ and $0.1^{0}C$ $min.^{-1}$ from $-30^{0}C$ to $33^{0}C$ before being plunged into liquid nitrogen. This method is effective, but relatively slow and involves a change of cooling rate.

2) Several laboratories have shown that good survival is obtained if embryos are plunged into liquid nitrogen from a temperature between $-20^{0}C$ and $-45^{0}C$ after cooling at $0.3^{0}C$ $min.^{-1}$ (Bilton 1980, Lehn-Jensen 1982, Elsden et al 1982).

3) One laboratory has obtained good survival when embryos were held at $-40^{0}C$ for 30 minutes before plunging, after being cooled at $0.3^{0}C$ $min.^{-1}$

4) Two-step cooling. Embryos were transferred from $-7^{0}C$ to an ethanol bath at $-30^{0}C$ for 30 minutes before being plunged into liquid nitrogen.

While a considerable proportion of embryos frozen by each of these methods survived, there are no direct comparisons to permit selection of the most effective method.

(iii) Warming Rate

There has been only one study of the effect of warming rate on embryo survival (Elsden *et al* 1982). The method of "rapid warming" employed involved shaking the container in water at 20°C - 25°C (Willadsen 1977, Bilton 1980) or 37°C (Lehn-Jensen *et al* 1982, Renard *et al*, 1982). However it must be appreciated that different test tubes and straws have been used and that different volumes of samples are employed, and that these factors will influence the warming rate of the sample. In the only direct comparison, it was found that it is better to thaw the sample at 37°C (Elsden *et al* 1982).

(iv) Cryoprotective Agents

Although there have been few direct comparisons (Bilton, 1980, Willadsen 1980, Elsden *et al* 1982) there has been a gradual change from the use of DMSO to the use of glycerol. The subjective opinion appears to be that glycerol is generally as effective, but that it is particularly protective in systems involving rapid thawing.

It was established that 1-2 propanediol is comparable to DMSO for the cryopreservation of bovine embryos (Renard *et al* 1982). From 20 embryos frozen in a solution of 2 M propanediol in PBS and stored for up to three months in liquid nitrogen, 65 per cent were apparently normal after thawing, 40.8 per cent hatched *in vitro* after 12-20 hours of culture producing five live calves after cervical transfer. The survival rate was comparable to one obtained with embryos frozen in 1.5 M DMSO.

Some survival was also obtained with ethyleglycerol, but survival was greater with glycerol (Elsden *et al* 1982). In order for a compound to be considered more effective than glycerol, it would either have to

offer more protection, or be easier to remove than glycerol.

(v) Methods of Dilution

After freezing and thawing, dilution of the cryoprotective agent out of the medium is essential for the development of frozen-thawed low embryos (Willadsen *et al* 1978). Protectants can be removed gradually after thawing in order to reduce injury from osmotic effect (shock). A solution containing 1.5 M DMSO has an osmolarity of 1.5 osmols or higher and during dilution this is reduced to normal physiological level of around 0.3 osmols (Whittingham 1980). When an isotonic solution is added to a medium containing cryoprotectant and embryos, an osmotic shock occurs as a result of influx of water into the hypertonic cells. This results in an increase in cell volume with some destructive consequences. This is due to the lower permeability of the common permeable protectants (DMSO and glycerol) to cell membrane than water (Ronald and Thomas, 1981). The degree of such swelling during dilution depends upon the amount of the cryoprotectant within the cells, the rapidity with which it can move out of the cell and the method of dilution used.

An alternative system of dilution employs the osmotic effects of an extracellular compound to remove cryoprotective agents from the cells. Embryos can be held in a hypertonic sucrose solution at room temperature for 30 minutes after thawing to permit diffusion of the permeable protectant out of the cell (Leibo and Mazur, 1978). This non permeable solute (sucrose) maintains a constant external osmolarity and has sufficient osmotic pressure to prevent injurious cellular swelling during the efflux of the permeating protectant. Such a hyperosmotic solution was also observed to shrink the cells and pump

out the permeating cryoprotectant out of the cells (Leibo and Mazur 1978).

Niemann et al (1982) compared the method of step-wise dilution of 10 per cent glycerol at room temperature at 10 minute intervals with a glycerol-sucrose gradient in PBS dilution method. They found that survival rate improved significantly after diluting with sucrose-glycerol gradient as compared with the step-wise glycerol dilution (83.3 versus 53.3 per cent). During the conventional step-wise glycerol dilution they observed that embryos were irreversibly destroyed by a drastic osmotic change which damaged the membranes of the blastomere and such damage was not observed when sucrose-glycerol gradient was used. Renard et al (1982) also reported the beneficial effects of using a sucrose gradient (PBS + .5 M sucrose) instead of step-wise dilution.

(vi) Integration of these Procedures

Whereas the initial methods of freezing and thawing embryos involved handling the embryos, on a microscope through several different media, protracted cooling and warming and further manipulative procedure after thawing, the procedures are now being greatly simplified. The rapid method of cooling and warming are now well established, but more recently a novel system of dilution and transfer of the embryos has been introduced. Embryos are frozen and thawed in the straws which are employed for the freezing of semen, but inside the straw are two discrete bubbles of fluid. In one is the embryo in PBS containing glycerol, whereas the other portion of fluid is PBS containing sucrose. After freezing and thawing, the straw is shaken to mix the fluids and initiate the removal of glycerol from the

embryo. It is then possible to transfer the embryos to a recipient non-surgically (Renard et al 1982, Leibo, 1982). While the pregnancy rates obtained which are around 50 per cent is very encouraging, it is clear that further improvements can be obtained. Such improvement will depend upon optimisation of the method of cooling, thawing and dilution.

The objectives of the present experiments were to make a direct comparison of three systems of cooling and to investigate the possibility of freezing embryos in the presence of sucrose and glycerol.

2-5 Assay

The methods which are being used to evaluate the quality of mammalian embryos after collection or culture are as follows:-

- Morphological appearance
- Their ability to develop in culture
- Their ability to develop in laboratory animals
- Retention of fluorescein dye
- Measurement of respiratory quotient
- Staining with some vital stain

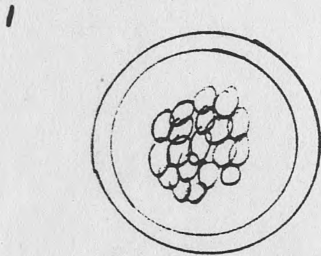
Of these methods, morphological appearance is the most widely used. However, beyond the differentiation of fertilized from unfertilized egg, it depends very much on experience and is subjective. Broadly, embryos are classified into normal, abnormal and degenerates. The morphology of normal eggs 1-cell to hatched blastocysts is shown in Fig. 5. Abnormal embryos are those with either irregular cell mass, debris, loose blastomeres, vacuoles in cytoplasms with cracked or empty zona. (Fig. 6).

In mouse, the number of frozen-thawed embryos which appeared morphologically normal immediately after recovery, was highly correlated with the ability to continue development to the blastocyst stage in culture and with continued development to offspring *in vivo* (Whittingham *et al* 1972). After morphological assessment, Bilton (1980) recorded a survival rate of 57 and 15 per cent for the morphologically normal and abnormal embryos respectively. He therefore concluded that morphological assessment could be a useful means of predicting the quality of embryos after freezing and thawing, but that there are exceptions.

In 1979, Bilton (1979) reported that 8-9 day old embryos losing or devoid of zona pellucida before freezing, did not during post thaw

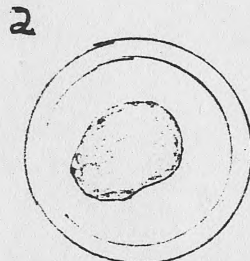
Fig. 5 Morphology of normal embryos.

1



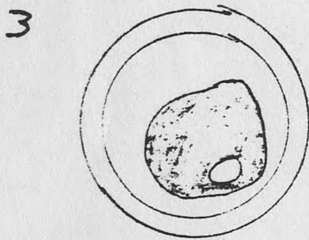
16-32 cell embryo
not compacted

2



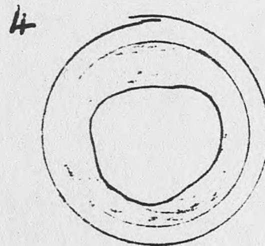
Morula (32 cells +)

3



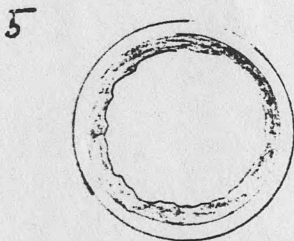
Early blastocyst

4



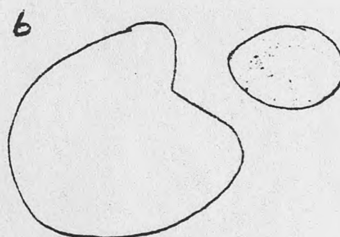
Blastocyst

5



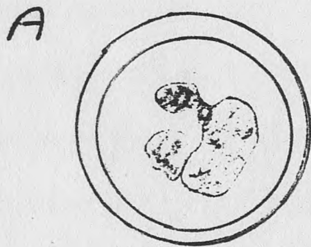
Expanded blastocyst

6

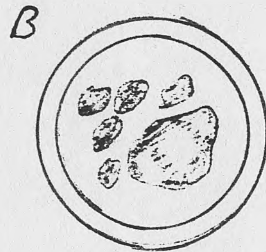


Hatched

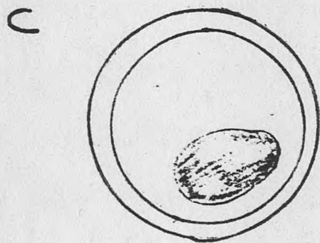
Fig. 6 Morphologically abnormal embryos



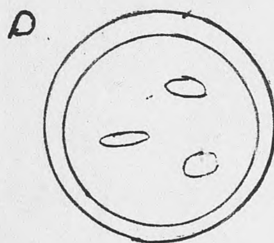
Morula with
1-2 fragments



Morula with more
than 2 fragments



Morula with debris



Disintegrated

culture and also did not result in pregnancy after transfer. It was also reported that hatched blastocysts (11-13 day old) did not survive freezing and thawing (Trouson _et al_, 1978). However, Tervit and Elsden (1981) showed that 10 day old hatched blastocysts readily survived freezing and developed during culture but had low survival after transfer. They suggested that this failure to develop may be due to lack of an intact zona and its protection in utero, or the embryo may have been damaged during freezing or thawing, the damaged zona being an obvious indication of such trauma. After transfer of 10 day old bovine embryos, Tervit and Elsden (1981) recorded a pregnancy rate of 10 of 14 and o of 6 embryos transferred with normal and damaged zona pellucida respectively.

Shea _et al_ (1976) studied how direct assessment of embryos at the time of collection related to subsequent pregnancy by scoring embryos on the basis of compactness, symmetry and density of blastomeres with a score of 1 representing a very poor looking and 5 excellent looking spacemen. Although highly scored embryos produced higher survival rate than the poorly rated ones, the result at times was variable. He noted that sometimes poorly rated groups could result in high pregnancy rate and this makes this method very difficult to discard the potentially valuable embryos as bad.

The quality of frozen-thawed embryos can also be evaluated by staining a representative of groups of embryos. The number and appearance of nuclei and state of cell membrane are being used as a criterion of normality. Thadani _et al_ (1982) described a staining procedure for evaluating frozen-thawed mouse embryo. The embryos are stained using 0.02 per cent neutral red and 0.5 per cent tryphan blue in modified Whittens medium. Live cells stain red and dead cells

blue with this procedure.

Rotman and Paper Master (1966) reported that fluorescein diacetate (FDA) could be used to assess the viability of cells, because living mammalian cells accumulate fluorescein when exposed to FDA (fluocromasia) The model proposed by Rotman and Paper Master (1966) to explain fluocromasia is that - FDA being non-polar, can readily pass into the cell where it is hydrolysed to yield fluorescein. The polar fluorscein cannot readily cross the cell membrane and therefore accumulates intracellularly. This test is therefore a means of measuring both estrases activity and cell membrane integrity. The incubation time required is short (1 min. - Rotman and Paper Master, 1966). The ability of the mouse embryos to retain the fluorescein following freezing and thawing is highly correlated (0.96) with their ability to develop in vitro (Jakowski 1977).

The observation of pulse-like contraction by the trophoblastic layer of embryos was suggested by McLaren (1972) as a means of evaluating the viability of embryos. However, the complexity of the test outweighed its practical application.

The ultimate test of the survival of frozen-thawed embryos is the production of viable young after transfer to foster mother. However, the direct transfer method of post-thawed embryo assessment is too costly and may require considerably long time because of the long gestation period of some species (e.g. 9 months for the cow). Therefore embryo development in vitro is the most common evaluation criterion. Most mammalian embryos will develop at least for 24 hours in vitro. This therefore might facilitate the identification of non-viable or damaged embryos and therefore only embryos showing viability can then be transferred to foster mothers.

Whittingham (1977) showed that the survival rates of frozen-thawed mouse embryos cultured for 20-24 hours were 30 per cent greater than that transferred to recipient immediately after thawing. He also found that the proportion of those embryos surviving freezing and thawing as indicated by their development in culture, which subsequently developed into young after transfer to recipients is similar to the proportion of unstored embryos which subsequently developed after transfer to recipients.

Bilton and Moore (1979) after culture and transfer of cow embryos found that apart from one recipient which received one embryo which had and two which had not developed in culture, no recipient which received embryos which failed to develop in culture subsequently calved, whereas 10 of 23 which received embryos which had developed in culture calved. This indicates that a short period of culture could provide a reliable and rapid method of assessing the viability of frozen-thawed embryos. At present, PBS containing foetal calf serum appears the culture system of choice for assessing the viability of embryos during the first 24 hours post thaw.

Although a considerable degree of success has been achieved using culture to assess the viability of mouse embryos, the development of embryos of other mammalian species (e.g. cow and sheep) over a prolonged period in culture has not been successful. This is because of the limited amount of information that is known on the nutritional requirement for growth of these embryos in vitro. Nevertheless, development through one to two cleavage division in vitro can provide sufficient information on the success of the freezing and thawing procedure (Whittingham 1980).

It has been noted that degenerative changes not evident after warming may appear after incubation in rabbits oviduct (Trounson _et al_ 1978). Therefore this might serve as an alternative means of assessing the viability of frozen-thawed embryos of cows and sheep.

3 Materials and Methods

Animals

Thirty cross-bred bulling heifers of unknown age were used as donors of the embryos. The embryo recovery and freezing work took place at the Dryden Field Laboratory (Roslin) between July and September 1982. The sources and composition of the drugs used are given in Appendix IV.

Oestrous Synchronisation

The oestrous cycles of all the animals used were synchronised using a prostaglandin analogue (Estrumate). Two doses were given to each animal eleven days apart, and each dose containing 500 mg cloprostenol in 2 mls was given intramuscularly.

Super Ovulation

The super ovulation was achieved using 2,500 or 5,000 i.u. Pregnant Mare Serum Gonadotrophin (PMSG) in 4 mls of saline intramuscularly between 8-16th day after the observation of heat. Forty-eight hours later a dose of cloprostenol or prostaglandin sodium salt (Lutalyse - Upjohn) was given. Animals were then mated naturally by a proven bull at the observed heat. Some animals received an injection of serum from sheep which have been immunised against PMSG.

Embryo Recovery

The embryos were collected non-surgically on day 7-8 after mating by the following method:-

- The animals were sedated by intravenous administration of acetypromazine at a dose rate of 0.05 mg/kg body weight.
- 20 mls of Buscopan were given intramuscularly as an analgesic and antispasmodic.
- Epidural anaesthesia was achieved by injecting 7-10 mls of Lignocaine (C-vet) between second and third intercoccygeal space. This is to prevent straining.
- A three-way catheter was used for the collection and the procedure described by Sugie et al (1972) was adopted. The flushing medium was 250 - 300 mls of modified Dulbecco's phosphate buffer (DPBS). (Whittingham 1971) supplemented with bovine serum albumen at 4 mg/mls (Appendix iii). The flushings were collected in a boiling test-tube in a cushion rack. Embryos were then allowed 5 to 10 minutes to gravitate and the supernatant removed. The remaining contents of the test-tube were transferred into an embryonic dish for embryo retrieval under microscope. The embryos were then morphologically assessed and graded according to the guide given in Fig. 5 and 6. using a 100 X magnification.

Freezing and Thawing

Embryos were held at room temperature (20°C) for up to 4 hours (until required). The addition and removal of cryoprotective agents were carried out at room temperature. The embryos were frozen and thawed individually in 50 x 10 mm soda glass test tubes containing 0.2 - 0.3 ml medium. After addition of the agent (to be specified) the tubes were transferred directly to a Planar controller PTC 101 pre-cooled to -7°C. The suspending medium was nucleated at -7°C by

the application of forceps which had been pre-cooled in liquid nitrogen to the outside of the tube until crystallisation was observed. The tubes were then cooled as specified, before being placed in plastic holding containers and plunged into liquid nitrogen.

The samples were thawed by agitating the tubes in a water bath at 37°C until the last ice disappeared. The tubes were then left at room temperature.

Assessment of Embryos

Each embryo was morphologically assessed at the time of recovery, immediately after removal of the cryoprotectant and after culture.

Culture

To confirm or otherwise the morphological assessment made after thawing or recovery, the embryos were put in a 50 X 10 mm soda glass test tube containing DBPS, 20 per cent foetal calf serum and antibiotics (Penicillin 0.06 mg/litre, Streptomycin 0.05 mg/litre). They were then placed in an incubator at 38°C for 18-24 hours. The embryos which continued to develop were judged to have survived the treatment they were subjected to during freezing.

Experimental Treatments

Experiment I

An experiment was carried out to compare three systems of cooling. All embryos were cooled at 0.3°C min. to -33°C before being cooled in three ways.

a) Direct plunge into liquid nitrogen from -33°C.

b) Held at -33°C for 30 minutes before being plunged into liquid nitrogen.

c) Cooled at 0.1°C min. from -33°C to 36°C and then plunged into liquid nitrogen.

The embryos were frozen in the presence of 10 per cent glycerol which was added in three steps with 10 minute intervals. After thawing the glycerol was removed in six steps with 5 minute intervals. (Willadsen, 1977).

Experiment 2

A pilot trial was carried out to confirm whether embryos could tolerate direct transfer into and outof a medium containing 10 per cent glycerol and either 0.25 M or 0.5 M sucrose. A comparison was made with embryos to which 10 per cent glycerol had been added in three steps. The embryos were allowed to equilibrate for 10 minutes before transfer to 1 ml of PBS.

Experiment 3

In two treatment groups, embryos were frozen and thawed in the presence of 10 per cent glycerol and either 0.25 M or 0.5 M sucrose. In the first group, the embryos were transferred directly to medium

containing 10 per cent glycerol and 0.5 M sucrose and after seeding were cooled at 0.2°C min. The embryos were plunged into liquid nitrogen from -15, -20, -25 or -30°C. After thawing the glycerol and sucrose were removed by direct transfer of the embryos into 1 ml PBS.

Experiment 4

Embryos were cooled to -33°C at 0.3°C min. and plunged into liquid nitrogen in the presence of 10 per cent glycerol and either 0.25 M or 0.5 M sucrose. The protective agents were removed either by direct transfer to 1 ml PBS or by initial transfer to 1 ml PBS containing sucrose (at the same concentration as was present during freezing) before transfer to PBS after 10 minutes equilibration time.

4 - Results

The effects of the three cooling procedures on the survival of embryos are presented on Table Ia. The direct plunging of embryos into liquid nitrogen at -33°C gave the same survival rate as plunging at -36°C after being cooled at 0.1°C min.$^{-1}$ from -33°C (33.3%). By contrast none of the embryos survived when held for 30 minutes at -33°C before being plunged into liquid nitrogen. As each embryo was frozen individually it is possible to assess whether or not embryo quality influenced the survival rate (Table 1b). Although a greater proportion of normal embryos survived freezing, the difference was not significant (31 versus 15 per cent χ^2 0.5 1df.).

The result of the pilot experiment confirmed that embryos can survive when transferred into and out of media containing 10 per cent glycerol and either 0.25 or 0.5 M sucrose. When the embryos were transferred either directly or step wise into such media the overall survival rate was 87 per cent (Table 2). There was no significant difference between the effect of the two levels of sucrose (concentration) on the survival rate.

In view of the fact that embryos could tolerate direct transfer into medium containing sucrose and glycerol, in experiment 3, embryos were frozen and thawed in the presence of 10 per cent glycerol and 0.5 M sucrose. After seeding, they were cooled at 0.2°C min.$^{-1}$ and plunged into liquid nitrogen at -15, -20, -25 and -30°C. After thawing glycerol and sucrose were removed by direct transfer of the embryos into 1 ml PBS. None of the embryos survived plunging at any of the 4 sub zero temperatures (Table 3).

In a further experiment, embryos were cooled in the presence of 10 per cent glycerol and either 0.25 or 0.5 M sucrose at 0.3°C min.$^{-1}$ to

$-33^{o}C$ (Table 4). The removal of the protectant was carried out in two different ways, either by direct transfer of embryos to PBS, or by transfer to a medium containing sucrose at the same concentration for 5 minutes before being transferred into PBS. Out of the total of 12 embryos frozen and thawed, none survived irrespective of either method of glycerol addition or sucrose concentration.

Table Ia

The survival of embryos cooled in three cooling methods with regard to plunging temperature and holding time

Cooling system	plunging from - 33°C	plunging from - 33°C after holding for 30 min.	plunging from -36°C after being cooled from -33 at .1°C min
No. of embryos frozen and thawed	21	9	12
No. developed in culture after thawing (%)	7 (33.3)	0	4 (33.3)

Table 1b

Survival of embryos cooled in three cooling system with regard to their quality before and after freezing

Cooling system	plunging at 33°C	plunging at -33°C after 30 min.holding	plunging at -36°C after being cooled at 1°C min.$^{-1}$ from -33°C.
Quality of embryos before freezing			
Normal	13	7	9
Grade 2	8	2	3
Quality of embryos after freezing			
Normal	6	0	3
Grade 2	1	0	1

Table 2

Survival of embryos after direct or step wise transfer into a medium containing 10 per cent glycerol and either 0.25 or 0.5 M sucrose

Medium	PBS + 10% glycerol (Direct addition)		PBS + 10% glycerol (step wise addition)
	Either 0.25 M sucrose	or 0.5 M sucrose	+ 0.5 M sucrose
No. of embryos frozen and thawed	4	5	5
No. developed after freezing (% of total frozen)	3 (75)	5 (100)	4 (80)

Table 3

Survival of embryos cooled in the presence of 10 per cent glycerol and 0.5 M sucrose at 0.2°C min. to various plunging temperatures. (C°C)

Plunging temperatures	- 15	- 20	- 25	- 30
No. of embryos frozen and thawed	3	3	3	3
No. developed in culture after thawing	0	0	0	0

Table 4

Survival of embryos cooled at 0.3°C min. to −33°C in the presence of 10 per cent glycerol and either 0.25 or 0.5 M sucrose and diluted either in 3 or 4 steps

Sucrose concentration	0.25 M		0.5 M	
Dilution method	* 3 steps	** 4 steps	* 3 steps	** 4 steps
No. of embryos frozen and thawed	3	3	3	3
No. of embryos developed in culture after thawing	0	0	0	0

* 3 steps – by direct transfer of embryos to PBS after thawing.

** 4 steps – by transfer to medium containing sucrose at the same concentration for five minutes before being transferred to PBS.

5 Discussion

The overall survival of embryos cooled with three methods of freezing was very poor as only 33 per cent of those plunged directly at $-33^{o}C$ and those plunged at $-36^{o}C$ after being cooled at $0.1^{o}C$ min. from $-33^{o}C$ survived. On the other hand, out of the twelve embryos held for 30 minutes at $-33^{o}C$ before plunging into liquid nitrogen, seven out of which were normal before freezing, none survived the freezing procedure. Several reasons could be suggested to account for the low survival. It may be that the embryos in these experiments were of poor quality because of the experimental methods of super ovulation, and it is known that embryo quality can influence survival (Willadsen _et al_ 1978). In these experiments all fertilized embryos which were recovered were frozen and thawed whereas in other reports only normal blastocysts and morulae were selected for freezing and thawing (Elsden _et al_, 1982, Niema _et al_, 1982, Farrad and Elsden, 1982 and Lehn-Jensen and Torvan Greve, 1982). The present results confirmed earlier observations that embryos of lower quality were less likely to survive. Nevertheless, it must be appreciated that only 40.9 per cent of the embryos judged to be normal before freezing and thawing, survived when plunged immediately from $-33^{o}C$ or after cooling to $-36^{o}C$ at $0.1^{o}C$ min.

It may be that the freezing procedure has been altered without full appreciation of the effect of any changes. In these experiments, embryos were plunged directly from temperature to $-7^{o}C$ rather than being cooled to this temperature at $0.1^{o}C$ min. (e.g. Willadsen, 1977). Although other groups have also begun to transfer directly to $-7^{o}C$ without an apparent decrease in survival (e.g. Bouysson and Chupin 1982, Renard _et al_ 1981), no direct comparison of the methods has been made.

Other less obvious changes on procedures may have been made. Similarly it may be that the chemicals used to make up the media were not satisfactory.

The sensitivity of embryos to different cooling and thawing rates has already been established (Whittingham, 1977), and the relationship between plunging temperature or degree of dehydration and thawing rate has also been shown (Bilton, 1980). The survival of embryos in this experiment when cooled at 0.3^{o}C min.$^{-1}$ to either -33°C or continued to -36°c at 0.1^{o}C min.$^{-1}$ before plunging into liquid nitrogen is consistent with the findings of Willadsen(1977), and Polge and Willadsen,(1978). Both reports indicated that rapid thawing of embryos could be successful if slow cooling is terminated between -30°C and -5°C prior to plunging into liquid nitrogen.

It is not clear whether or not a period of cooling at 0.1^{o}C min. improves survival. Our result showed that there was no significant difference between the direct plunging at -33°C and those plunged at -36°C after slower cooling (0.1^{o}C min.) from -33°C. The survival rate was lower (33.3 per cent) than that reported in some experiments (e.g. Lehn-Jensen, 1982 and Elsden et al, 1982), but it is in agreement with the findings of Massip et al, (1979). Elsen et al (1982) noted a significant improvement in pregnancy rate when cooling was decreased from 0.3^{o}C min. to 0.1^{o}C min. from 35°C. Niema et al,(1982) also reported a survival rate of 70 per cent when embryos were cooled to 28°C at 0.3^{o}C min. and then to -35°C at 0.1^{o}C min. followed by rapid thawing after storage in liquid nitrogen.

The failure of embryos to survive when held at -33°C for 30 minutes is consistent with the findings of Cockroft (1981). It could either be due to injury caused by excessive exposure of embryos to high

concentration of solute at a relatively high temperature (solute effect) or due to excessive dehydration aggravated by rapid thawing. Contrary to our findings, Bouysson and Chapin (1982) reported 83 per cent survival for the embryos held at -30^{0}C for 30 minutes after being cooled at 0.3^{0}C $min.^{-1}$ from -7^{0}C. They reported that there was no significant difference between the embryos held at -30^{0}C for 30 minutes and those directly plunged into liquid nitrogen at -30^{0}C. However, they used only blastocysts whereas we used a combination of morulae and blastocysts so may be if we had used blastocysts of the same quality, we would have got a better result. Elsden et al (1982) also reported a pregnancy rate of 23.4 per cent for the embryos cooled at 0.3^{0}C $min.^{-1}$ to -33^{0}C and held for 30 minutes before being plunged into liquid nitrogen. Also in their case only excellent to good embryos were used.

Smorag et al (1981) cooled mouse embryos in the presence of 1.5 M glycerol at 0.3^{0}C to -40 and held them over periods ranging from 0 to 60 minutes before plunging into liquid nitrogen. It was found that the survival rate on rapid thawing (36 ^{0}C $min.^{-1}$) improved from 29.4 per cent when plunged directly to 37 per cent when embryos were held for 45 minutes before plunging into liquid nitrogen. Also it has been reported that morulae to blastocysts stages of bovine embryos survived when cooled at 0.4^{0}C $min.^{-1}$ to -30^{0}C, held for 30 minutes, plunged into liquid nitrogen, thawed at approximately 150^{0}C min. and diluted with sucrose in a straw (Leibo, 1982). It was observed that fewer embryos survived when cooled at higher or lower rates or when cooled to a different temperature or when warmed at different rates.

The result of the preliminary experiment revealed that bovine embryos could survive when transferred into 10 per cent glycerol with either 0.25 or 0.5 M sucrose. The innocuouse effect of the combination

of sucrose and 10 per cent glycerol led to its usage as a diluent in the gradient dilution method. Nieman et al (1982) reported a survival rate of 83.5 per cent when glycerol-sucrose gradient was used for diluting out the glycerol used as a cryoprotectant. The innocuous nature of glycerol-sucrose combination to embryos noted in our experiment is consistent with the findings of Renard et al (1982) who established that bovine embryos could survive short time exposure to 1 per cent or long time exposure to 0.25 or 0.5 M sucrose.

While the innocuous effect glycerol-sucrose combination as a diluent for the permeating agent is well documented at room temperature (Nieman et al, 1982, Renard et al 1982, and Leibo, 1982), it is surprising to find that it failed to protect the embryos against the injurious effect of the sub-zero temperature. When embryos were cooled either at 0.2 or 0.3°C min.$^{-1}$ to various sub-zero temperatures in the presence of 10 per cent glycerol and either 0.25 or 0.5 M sucrose none survived. The method of addition or dilution of 10 per cent glycerol in and out of the media did not affect the result. Although the number of embryos used was small in both experiments (3 and 4), it is sufficient to conclude that a combination of 10 per cent glycerol and either 0.25 or 0.5 M sucrose failed to protect the embryos against freezing injury.

Since our preliminary experiment indicated that bovine embryos could survive a combination of glycerol and sucrose at room temperatures, and other workers (Nieman et al, 1982, Leibo, 1982 and Renard et al,1981) have shown that it could be used as a diluent at room temperature, then the failure of embryos to survive when frozen in this compound could be due to over dehydration worsened by rapid thawing. An experiment employing a higher rate of cooling or slower warming should be done to investigate the main cause of the injury.

6 Conclusion

It is difficult to draw valid comparison between the reports of different laboratories since conditions and procedures for freezing, thawing and dilution vary greatly. Nevertheless, the review of the literature shows that high survival is being obtained in several laboratories.

The overall survival in these experiments was low and emphasised the critical influence of embryo quality and careful laboratory procedure on survival after freezing and thawing. The death of all embryos which were held for 30 minutes at $-33^{o}C$ confirms the damaging effect of holding for an inappropriate length of time at sub-zero temperatures and suggests that care must be taken when employing methods of freezing which involve a holding period.

Our preliminary experiment confirmed that bovine embryos can tolerate a medium containing 10 per cent glycerol and either 0.25 or 0.5 M sucrose at room temperature. However, freezing embryos in such media is not compatible with survival. The death of these embryos could be associated with excessive dehydration due to hyper osmotic effect of the medium, so an experiment employing either a higher cooling rate or slower thawing rate should be done to study the mechanism of injury involved.

APPENDIX I

FREEZING AND THAWING PROCEDURE USED BY WILMUT (1972)

The tubes containing the samples were transferred from room temperature to crushed ice and after 15 minutes to a seeding bath which was cooled to a temperature approximately $2^{o}C$ below the freezing point of the medium. Ice formation was induced by seeding with ice crystal and 5 minutes later the samples were placed in the cooling vessels pre-cooled to the temperature of the seeding bath and cooling commenced at various rates to $-70^{o}C$ as follows:

Cooling rate (-10 to $-70^{o}C$)	Container
$0.07^{o}C\ min^{-1}$	An evacuated silvered flask, internal measurements 100 x 200 mm containing 850 ml ethanol.
$0.22^{o}C\ min^{-1}$	An evacuated unsilvered flask, internal measurements 100 x 200 mm containing 400 ml ethanol.
$0.67^{o}C\ min^{-1}$	400 ml double walled beaker, internal measurements 80 x 100 mm containing 150 ml ethanol.
$1.2^{o}C\ min^{-1}$	Empty evacuated unsilvered flask, internal measurements 100 x 200 mm.
$4.7^{o}C\ min^{-1}$	400 ml double walled plastic beaker containing 150 ml ethanol.
$23^{o}C\ min^{-1}$	Empty 250 ml pyrex beaker.
$80^{o}C\ min^{-1}$	25 x 150 mm pyrex tube pre-cooled in liquid nitrogen.
$690^{o}C\ min^{-1}$	Tubes placed directly in liquid nitrogen.

The warming rates which were obtained when the samples were taken from liquid nitrogen and placed in the containers.

Warming rate (-70 to $-10^{o}C$)	Container
$1.1^{o}C$ min	1 litre pyrex beaker containing 600 ml ethanol cooled to $-70^{o}C$. The beaker was allowed to warm in air.
$12^{o}C$ min	16 x 125 mm pyrex tube which had been cooled in liquid nitrogen. The tube was allowed to warm in air.
$60^{o}C$ min	Tubes shaken in air at room temperature.
$360^{o}C$ min	Tubes shaken in water at $37^{o}C$.

APPENDIX II

COOLING AND THAWING PROCEDURES USED BY WHITTINGHAM ET AL (1972)

Cooling

1) Ten to forty embryos in 0.00 ml of medium were pipetted into tubes containing 0.1 ml of PBS.
2) The samples were cooled to $0^{o}C$ and DMSO or glycerol was added.
3) After 15 minutes the samples were transferred to a bath at $-35^{o}C$ to $4.5^{o}C$ and seeded 2 minutes later with a minute ice crystal.
4) After another 5 minutes, they were transferred to baths cooling at $0.3^{o}C$ to $4^{o}C\ min^{-1}$.
5) Samples were cooled to -70°, 196°, or $-269^{o}C$, kept at these temperatures for 1 minute to 192 hours.

Thawing

The samples were thawed in four different ways.

1) A $35^{o}C$ water bath for 35 seconds (rate $450^{o}C\ min^{-1}$)
2) An ice bath (rate $215^{o}C\ min^{-1}$.)
3) Air at room temperature (rate $25^{o}C\ min^{-1}$)
4) 20 ml of ethanol in tubes (38 by 200 mm), the ethanol initially at $-110^{o}C$, warming by contact with room temperature (rate $4^{o}C\ min^{-1}$).

APPENDIX III

THE COMPOSITION OF DULBECCO'S MODIFIED PHOSPHATE BUFFERED SALT SOLUTION

Modified Dulbecco's PBS ingredients mg l^{-1}

NaCl	8,000
KCl	200
$CuCl_2$	100
$MgCl_2 6H_2O$	100
Na_2HPO_4	1,150
KH_2PO_4	200
Glucose	1,000
Na pyruvate	36
Penicillin	6.0
Streptomycin	5.0

APPENDIX IV

THE SOURCES OF THE DRUGS AND MATERIALS USED IN THE EXPERIMENT

Estrumate is a synthetic prostaglandin analogue for use in cattle, is structurally related to prostaglandin F_2 (PGF_2). Each ml contains 263 mcg of Cloprostenol sodium BP (Vet) equivalent to 250 mcg cloprostenol. Solution also contains 0.1% w/v chlorocrosol BP as a bactericide. Dose 2 ml (500 g cloprostenol) i/m.

Imperial Chemical Industries Ltd.,
Pharmaceuticals Division,
Alderley House,
Alderley Park,
Macclesfield,
Cheshire, SK10 4T

Folligon contains serum gonadotrophin. Dose 2,500 i.u. per cow.

Intervet Lab. Ltd.,
Science Park,
Milton Road,
Cambridge. CB4 4BH

Acetylpromaline inj. BP (Vet) acepromaline maleate 10 mg/ml Dose 0.05 mg/Kg i/m or slow i/v.

Berk Pharmaceuticals Ltd.,
Catteshall Lane,
Godalming,
Surrey. GU7 1LA

Buscopan compositum. Hyoscine N-butylbromide 4 mg/ml. Metamizole 500 mg/ml. Dose 20 ml i/m.

Crown Chemical Company Ltd.,
Lambhurst,
Kent. TN3 8DJ

Lignocaine (C-vet) Lignocaine Hydrochloride B.Vet.C w/v 2% w/v. Adrenalin acid tartrate BP w/v 0.0024% w/v. Chlorocretol 0.01% w/v. Sodium Metabisulphate BP 0.1% w/v. Sodium chloride 0.45% w/v. Water for injection BP to 100%.

C-Vet. Limited,
Minster House,
Western Way,
Bury St. Edmunds,
Suffolk, IP33 3SU

Catheter

Franklin Catheter,
Bovine Egg Collection Catheter,
J.G. Franklin & Sons Ltd.,
High Wycombe,
England,

Code E942200 Size 14Fr 20/30 ml

Planar Controller PTC 101

Planar Products Ltd.,
Windmill Road,
Sunbury-on-Thames,
Middlesex,
England.

Dulbecco's Phosphate Buffered Saline

Flow Laboratories,
Irvine,
Ayrshire,
Scotland.

Foetal calf serum

Flow Laboratories,
Irvine,
Ayrshire,
Scotland.

Bovine serum albumin (fraction 5 or purer)

British Drug House (BDH),
Poole,
Dorset.

Glycerol analar

British Drug House (BDH),
Poole,
Dorset.

REFERENCES

AVERILL, R.L.W. and ROWSON, L.E.A. (1959) Attempts at storage of sheep ova at low temperatures. J. Agric. Sci., Cam. 52., pp 392 - 395.

BILTON, R.J. (1980) Preservation of embryos of the large domestic species: In The 9th International Congress on Animal Reproduction and Artificial Insemination. Vol. 11, pp 245-253 held 16th-20th June, Madrid, Spain, 1980.

BILTON, R.J. and MOORE, N.W. (1977) Successful transport of frozen cattle embryos from New Zealand to Australia. J. Reprod. Fert. 50; pp363-369.

BILTON, R.J. and MOORE, N.W. (1979) Factors affecting the viability of frozen stored cattle embryos. Aust. J. Biol. Sci., 32: pp 101-107 (1979).

BOUYSSOU B., and CHUPIN, D. (1982). Two-step freezing of cattle blastocysts with Dimethyl Sulphoxide (DMSO) or Glycerol. Theriogenology, 17: 2 pp 159-169.

COCKROFT, P.D. (1981) A dissertation in partial fulfilment of the requirements for the degree of Masters of Science in Tropical Animal Health and Production. University of Edinburgh, 1981.

DOEBLIN, G.F., and RINFRET, A.P. (1962). The influence of protective compounds and cooling and warming conditions on haemolysis of erythrocytes by freezing and thawing. Biochim. Biophys. Acta, 58, pp 449-458.

ELSDEN, R.P., SEIDEL, G.E., TAKEDA, T.Jr. and FARRAND, G.D. (1982). Field experiment with frozen-thawed bovine embryos transferred non-surgically. Theriogenology 17, No. 1 (1982).

FARRAND, G.D., ELSDEN, R.P. and SEIDEL, G.E. Jr. (1982) Effect of slow cooling of bovine embryos prior to plunging in liquid nitrogen. Theriogeneology, 17: No. 1 pp 88.

FARRANT, J. (1972). Human red cells under hypertonic conditions, a model system for investigating freezing damage. Dimethyl cryobiology 9 pp 131-136 (1972).

FARRANT, J. (1977) Water transport and cell survival in crybiological procedures. Phil. Trans. R. Soc. Lond. B., 278 pp 191-205.

FARRANT, J. (1980) General observations on cell preservation. In: Low Temperature Preservation in Medicine and Biology (eds. Ashwood-Smith, M.J. and Farrant, J.) Chap 1, pp 1-18, 1st edition (1980). Pitman Medical Ltd., 57 High Street, Tunbridge Wells, Kent.

JACKOWSKI, S., LEIBO, S.P. and MAZUR, P. Glycerol permeabilities of fertilized and unfertilized mouse ova. J. Exp. Zool. 212 pp 329-341 (1980).

JACKOWSKI, S.C. (1977) Physiological differences between fertilized and unfertilized mouse ova; glycerol permeability and freezing sensitivity. Ph. D. Thesis, The University of Tennessee, Knoxville, U.S.A.

LEHN-JENSEN, H. and TORBEN GREVE (1982). The survival of cow blastocysts frozen in 1.4 M glycerol after plunging between -15 and 10°C and rapid thawing. Theriogenology, No. 17 pp 95.

LEIBO, S.P. (1977) Fundamental Cryobiology of mouse ova and embryos. In: The freezing of mammalian embryos, CIBA Foundation, 52, (ed. Elliott, K. and Whelan, J.) pp 69-91. Elsevier, Amsterdam.

LEIBO, S.P. (1982) Transfer of frozen-thawed bovine embryos (Abst). Society for cryobiology annual general meeting. Houston, Texas, 27th July 1982.

LOVELOCK, J.E., (1953) The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. Biochem. Biophys. Acta, 11 pp 28-36.

LOVELOCK, J.E., (1954) Physical Instability and thermal shock in red cells. Nature, London, 173 pp 659-661 (1954).

MASSIP, A., VAN DER ZWALMEN, P., ECTORS, F., DECOSTER, R., DILETEREN, G. and HANZEN, C. (1979). Deep freezing of cattle embryos in glass ampules in French straws. Theriogenology; 12: pp 79-84.

LEIBO, S.P. and MAZUR, P. (1970) The freezing of biological systems. Cryobiology 168 pp 393-949.

MAZUR, P., MILLER, R.H. and LEIBO, S.P. (1974). Survival of frozen-thawed bovine red cells as a function of the permeation of glycerol and sucrose. J. Memb. biol. 15 pp 137-158.

MAZUR, P. (1977) Slow-freezing injury in mammalian cells. In: Freezing of Mammalian Embryos, CIBA Foundation, 52 (ed. Elliott, K. and Whelan, J.) pp 19-41. Elsevier, Amsterdam.

MAZUR, P., RALL, W.F. and RIGOPOULOS, N. (1982). Relative contributions of the fraction of unfrozen water and of salt concentration for the survival of slowly frozen-human erythrocytes. Biophy. J., 36 pp 653-675.

McLAREN, A. (1972). The embryo In: Reproduction in Mammals; Embryonic and foetal development Vol. 2. (Eds. Austin, C.R. and Short, R.V.) Ch. 1, pp 1-42. Pub. Syndics of the Cambridge University Press, Bentley House, 200 Euston Road, London, NV1 2 DB.

MERYMAN, H.T. (1968). Modified model for the mechanism of freezing injury in erythrocytes. Nature, London, 218: pp 333-336 (1968).

NEI TOKIO, (1977). Structure and function of frozen cells: freezing patterns and post-thaw survival. J. of micros, 112 pp 197-204.

NIEMAN, H., SACHER, B., SCHILLING, E., and SMIDT, D. (1982). Improvement of survival rates of bovine blastocysts with sucrose for glycerol dilution after a fast freezing and thawing method. Theriogenology; 17: 1 pp 102.

POLGE, C., SMITH, A.U. and PARKES, A.S. (1949). Revival of spermatozoa after vitrification and dehydration at low temperature. Nature, London, 164, 666.

POLGE, C. and SOLTYS, M.A. (1959). In: Recent Research in Freezing and Drying (Eds. A.S. Parkes and A.U. Smith Blackwells, Oxford (1959).

POLGE, C. and WILLADESEN, S.M. (1978). Freezing eggs and embryos of farm animals. Crybiology, 15, 370-373.

RENARD, J.P., OZIL, J.P., HEYMAN, Y. (1981) Cervical transfer of deep frozen cattle embryos. Theriogenology 15: pp 311-320 (1981).

RENARD, J.P., MEMEZO, Y. and HEYMAN, Y. (1982) Alternative tests to assess viability of bovine embryos. Theriogenology 17: pp 106.

RONALD, L.L. and THOMAS, W.J. (1981) An optimum method for the Introduction or removal of permeable cryprotectants: Isolated cells. Crybiology, 18 pp 32-48.

ROTMAN, B. and PAPERMASTER (1966). Membrane properties of living cells as studied by enzymatic hydrocysis of fluorogenic esters. Proc. Natn. Acad. Sci. U.S.A. 55; pp 134-141.

SHEA, B.F., HINES, D.J., LIGHTFOOT, OLLIS, G.W. and OLSON, S.M. (1976). The transfer of bovine embryos. In: Egg transfer in cattle (Ed. L.E.A. ROWSON) Commission of the European Communities, Luxemburgh 1976. Eur 5491, pp 145-152.

SHEA, B.F. (1981) Evaluating the bovine embryo. Theriogenology, 15 pp 31-42.

SMITH, A.U. (1952) Behaviour of fertilized rabbit eggs esposed to glycerol and to low temperatures. Nature, London. 170, pp 374-375.

SMOGAG ZOZISLAW, LUCYNAKATSKA and EDWARD WIERZCHOS (1981) Some factors affecting the viability of mouse and cattle embryos frozen to $-40^{\circ}C$ before transfer to liquid nitrogen. Animal Reprod. Sci. 4 pp 65-77

SUGIE, T., SOMA, T., FUKUMITTSU, S. and OTSUKI, K. (1972). Studies on the ovum transfer in cattle with special reference to collection of ova by means of non-surgical technique. Bull. Nat. Inst. Anim. Ind., 25, 27, pp 28-56.

SUGIE, T. (1965) Successful transfer of a fertilized bovine egg by non-surgical techniques. J. Reprod. Fert. 10 pp 197-201.

SUGIE, T., SEIDEL, G.E. and HAFEZ, E.G.E. (1980). Embryo transfer In: Reproduction in Farm Animals (ed. Hafez, E.S.E.) Ch. 29, p. 578. 4th Edition 1980. Pub. Great Britain, Balliere, Tindall, London.

TERVIT, H.R.,ELSDEN, R.P., and FARRAND, G.D. (1981) Deep freezing 7 to 8 and 10 to 11 day old cattle embryos. Theriogenology 15:1, pp 114.

THADANI, V.M., DARNELL, D.K., SEIDEL, G.E.Jr., and TAKED, T. (1982). Toxicity Evaluation of a vital stain procedure for Frozen-Thawed Mouse embryos. Theriogenology Jan. 1982 Vol. 17 No. 1 pp 110.

TRAN, P.T., and BENDER, M.A. (1960). Protecting mouse bone marrow by inorganic compounds during freezing and thawing. Proc. Soc. Exp. Biol. Med., 104, pp 388-390.

TROUNSON, A.O., WILLADESEN, S.M., ROWSON, L.E.A. and NEWCOMB, R. (1976). The storage of cow embryos at room temperature and at low temperature. J. Reprod. Fert., 46, 173-178.

TROUNSON, A.O., WILLADESEN, S.M. and ROWSON, L.E.A. (1976). The influence of In-Vitro culture and cooling on the survival and development of cow embryos. J. Reprod. Fert. 47, pp 367-370.

TROUNSON, A.O., BRAND, A. and AARTS, H.A. (1978) Non-surgical transfer of deep frozen bovine embryos. Theriogenology 10, pp 111-113.

TROUNSON, A.O., SHEA, B.F., OLLIS, G.W. and JACOBSEN, M.E. (1978). Frozen storage and transfer of bovine embryos. Journal of Animal Science, 47 No. 3, pp 677-681.

TSUNODA, V. and SUGIE, T. (1977). The effect of the freezing medium on the survival of rabbit eggs after deep freezing. J. Reprod. Fert. 50 pp 123-124.

WHITTINGHAM, D.G. (1971) Survival of mouse embryos after freezing and thawing. Nature, London, 233, pp 125-126.

WHITTINGHAM, D.G. (1974) The viability of frozen-thawed mouse blastocysts. J. Reprod. Fert. 37 pp 159.

WHITTINGHAM, D.G. (1977) Some factors affecting embryo storage in laboratory animals. In: Freezing of Mammalian Embryos, CIBA Foundation, 52, (Eds. Elliott, K. and Whelan, J.) pp 97-107. Elsevier, Amsterdam.

WHITTINGHAM, D.G. (1980) Principles of embryo preservation. In: Low Temperature Preservation in Medicine and Biology (Eds. Ashwood-Smith, M.J. and Ferrant, J.) Ch. 4 pp 65-83. 1st Edition (1980), Pitman Medical Ltd., 57 High Street, Tunbridge Wells, Kent.

WHITTINGHAM, D.G. and ANDESON, E. (1976) Ultra-Structural Studies of frozen and thawed 8-cell mouse embryos. J. Reprod. Fert., 48; pp 137-140.

WHITTINGHAM, D.G., LEIBO, S.P. and MAZUR, P. (1972). Survival of mouse embryos frozen to $-196^{o}C$ and $296^{o}C$. Science, 178, pp 411-414.

WHITTINGHAM, D.G. and WALES, R.G. (1969) Storage of two-cell mouse embryos In Vitro. Aust. J. Biol. Sci. 22 pp 1065-1068.

WHITTINGHAM, D.G. and WHITTEN, W.H. (1974). Long-term storage and aerial transport of frozen mouse embryos. J. Reprod. Fert. 36, pp 433-435.

WILLADESEN, S.M. (1977) Factors affecting the survival of sheep embryos during freezing and thawing. In: Freezing of Mammalian Embryos, CIBA Foundation 52, (Eds. Elliott, K. and Whelan, J.) pp 175-189. Elsevier Amsterdam.

WILLADESEN, S.M., TROUNSON, A.A., POLGE, C., ROWSON, L.E.A., and NEWCOMB, R. (1976). Low temperature preservatio n of cow embryos. In: Egg Transfer in Cattle (Ed. Rowson, L.E.A.) pp 117-124. Agriculture Research Service, E.E.C. Commission, Luxembourg.

WILLADESEN, S.M. and TISCHNER, M. (1978). Successful long distance transport of sheep embryos stored in liquid nitrogen. Bull. Acad. Po Sci: Cl 11 Ser. Sci. BIDI 26, pp 725-726.

WILLADESEN, S.M., POLGE, C. and ROWSON, L.E.A. (1978a) In vitro storage of cattle embryos. In: Current topic in Vet. Med. Vol 1 Control of Reproduction in the Cow. Ed. Screenan, J.M.) pp 427-436. Pub. Commission of European Committees, Directorate General Scientific Technical Information and Information Management, Luxembourg.

WILLADESEN, S.M., POLGE, C. and ROWSON,L.E.A. (1978b) The viability of deep frozen cow embryos. J. Reprod. Fert., 52 pp 391-393.

WILMUT, I. (1972) The effect of cooling rate, warming rate, cryoprotective agent and stage of development on survival of mouse embryos during freezing and thawing. Life Sci. 11, 1071-1079.

WILMUT, I., POLGE, C. and ROWSON, L.E.A. (1975). The effect on cow embryos of cooling to $20^{o}C$, $0^{o}C$ and $-196^{o}C$. J. Reprod. Fert., 45, pp 409-411.

WILMUT, I. and ROWSON, L.E.A. (1973) Experiments on the low temperature preservation of cow embryos. Vet. Rec., 92, 686-690.