DEVELOPMENT AND EVALUATION OF CRYOPRESERVATION TECHNIQUES FOR BOVINE EMBRYOS

by

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SUMMARY

Several procedures for cryopreservation and the factors affecting cryopreservation of embryos are reviewed. A total of 228 embryos were non-surgically collected from superovulated cows. The embryos were then dehydrated in DMSO or glycerol by a 3-Step procedure or a timed interval titration procedure. They were frozen to -196 C and thawed in a 27 C or 37 C water bath and rehydrated by a 6-Step, 3-Step (sucrose) or 1-Step (sucrose) procedure. This yielded a 2 X 2 X 2 X 3 factorial treatment structure. Embryos were graded according to stage of development and were quality graded post-dehydration, post-thawing, and 10 min post-rehydration. Survival was based on development after 12 h in vitro. Neither the cryoprotectant (DMSO, glycerol), dehydration procedure (Timed Interval Titration, 3-Step), thaw temperature (27 C, 37 C), rehydration procedure (6-Step, 3-Step, 1-Step), number of d frozen, breed, sire or dam of the embryo nor the stage of development (early blastocyst, blastocyst, expanded blastocyst) had any effect on survival ($P>.10$). The only factor significantly related to survival was the initial quality grade of the embryo. Quality grade 1 and 2 embryos survived more often than quality grade 3 embryos ($P<.05$). Interactions indicated that the 3-Step rehydration procedure resulted in higher survival rates if the thaw temperature was 27 C ($P<.05$). Embryos more advanced in development and with better quality classifications survived more often ($P<.05$). Freezing in glycerol resulted in survival more often if the embryos were rehydrated by the 6-Step procedure versus the 1-Step procedure ($P<.05$). From post-dehydration to post-thawing, using DMSO as the cryoprotectant resulted in better quality scores post-

thawing (P=.02). For both intervals, post-dehydration to post-thawing, and post-thawing to post-rehydration the previous quality grade significantly affected the new quality grade (P<.01).

(Key Words: Bovine, Embryo, Cryopreservation, Sucrose)

INTRODUCTION

Costs for embryo transfer are high and today only justifiable to the purebred producer. The costs have been estimated to be between $2,000 and $3,000 for each 6-month old calf (Seidel and Seidel, 1978). Baker (1979) has estimated that one out of every 7 embryo collections has resulted in an excess of quality embryos for the recipients available. Cryopreservation of these embryos provides a means to avoid embryo and genetic wastage due to poor quality or unavailable recipients and can also aid in the international merchandising of cattle. The ease of movement and limited quarantines of frozen embryos allow an inexpensive means of transportation to remote regions of the world. To limit the potential for disease transmission caused by embryo movement it has recently been suggested that international health certification for frozen embryos be adopted (Waters, 1981).

Cryopreservation of rare breeds or blood lines that have special qualties such as resistance to disease would greatly reduce the costs of maintaining these breeds (Bradford and Kennedy, 1980). The ability to keep a wide genetic base that would aid in making a quick genetic change may be economically justifiable. The genetic change now possible with embryo transfer (McDaniel and Cassell, 1981; Cunningham, 1976; Bradford and Kennedy, 1980; Wilmut and Hume, 1978) would only increase with adequate cryopreservation techniques.

Cryopreservation of embryos can enable geneticists to prevent

genetic drift and maintain certain research lines which, conventionally, are expensive to maintain (Wilmut, 1976). It can also aid in testing for certain genetic traits such as syndactly (Leiopold and Peeples, 1981) and polydactly (Castelberry et al., 1981), or to discover the causes for early embryonic death embryonic death (Cartwright et al., 1980). The advantages of embryo storage expressed by Wilmut (1976) are to: 1. create embryo banks, 2. facilitate export, 3. create genetic controls, and 4. to conserve rare breeds.

The main objective of this research was to develop and evaluate a cryopreservation program for bovine embryos.

The specific goals were to:

1. evaluate DMSO and glycerol as cryoprotectants,
2. develop an efficient dehydration procedure.
3. evaluate thaw bath temperatures of 27 C and 37 C, and
4. determine if the incorporation of sucrose into the cryopreservation process is advantageous.

LITERATURE REVIEW

Factors Affecting Stage of Development and Freeze-Thaw Survival

As early as the first successful frozen-thawed bovine embryo it was noted that the stage of development of the embryo was important to survival (Wilmut and Rowson, 1973). The stage of development that results in optimum survivability of freeze-thawing seems to vary by species. In mice it is from the oocyte to the blastocyst stage (Maurer, 1978). In experiments with ova collected from pigs, at any stage of development from four cells up to the hatched blastocyst, none have survived cooling to 0.0 C (Polge, 1977).

Willadsen et al. (1978b), Trounson et al. (1976a) and Wilmut et al. (1975) found that preмorula stage bovine embryos are sensitive to cooling. Specific changes found by Mohr and Trounson (1981) are that cooling d 5 bovine embryos to 4 C disrupted the distribution of organelles within blastomeres as well as the spacial arrangement of the blastomeres. Schneider and Hahn (1979) demonstrated that only 1 of 9 morula stage embryos and 6 of 10 blastocyst stage embryos survived freeze-thawing. Later Lehn-Jensen (1980b) confirmed Schneider and Hahn's results by finding that an increased tolerance of bovine embryos to low temperatures coincided with compaction and blastulation of the embryo. In 1982 Farrand et al. found that in all cases, bovine embryos which had formed a blastocele exhibited higher survival rates than did the morula.

For the more advanced stages of development, Bilton and Moore (1979) and Mohr and Trounson (1981) revealed a high sensitivity to freezing. In Bilton and Moore's work, 8 of 19 d 7 embryos survived freezing, whereas none of 16 d 9 embryos survived freezing. Earlier Trounson et al. (1978b) had similar results using 7 and 8 d embryos versus 11 and 13 d embryos. Mohr and Trounson's research showed that d 13 bovine embryos were severely structurally damaged after freezing and thawing. The damage to the membranes was so severe that the cryoprotectant (DMSO) could not be removed before the embryos were fixed for electron microscopy, because a high proportion of the embryos disintegrated during the cryoprotectant removal.

The optimum stage of development appears to be the blastocyst. Trounson et al. (1978a) and Lehn-Jensen and Greve (1978) found that expanded blastocysts survived the rigors of freezing and thawing best, but Niemann et al. (1982a) found that d 7 early blastocysts survived and

resulted in pregnancies more often than d 7 morula or d 8 expanded blastocysts. They suggest that the desmosomes, tonofilaments and gap junctures, which first appear at the blastocyst stage and are responsible for hatching, are damaged to an extent that normal hatching cannot take place, resulting in lower survival rates.

Freezing Vessels

Early research with freezing embryos used ampules or test tubes as the freezing vessels (Wilmut and Rowson, 1973; Wilmut et al., 1975). Since the final objective of freeze-thaw embryo transfer research is to perfect a 1-step transfer method similar to A.I., a more suitable freezing vessel to obtain this goal was researched. Current procedures for nonsurgical embryo transfer call for embryos to be loaded into a .5ml or .25ml French straw for transfer via Cassou gun to the tip of the uterine horn (Rowe et al., 1980; Greve and Lehn-Jensen, 1979; Greve, 1980; Tervit et al., 1980; Curtis et al., 1981).

Massip et al. (1980) found the use of plastic straws easy and practical. Since plastic straws are the popular means of transfer, comparisons were made between freezing embryos in plastic straws and glass ampules. Lehn-Jensen (1980b) found that 3 of 8 (38%) embryos frozen in plastic straws survived freezing compared with 9 of 20 (43%) frozen in ampules. Massip et al. (1979) had overall survival rates of 36% for ampules and 31% for straws (94 embryos total). But Kennedy et al. (1983) had a greater proportion of embryos develop in culture after freezing in straws than in ampules. Bouyssou and Chupin (1982a) found that after 6 h in culture there was no significant difference in viability between the embryos frozen in straws or ampules. This finding was also concluded by Massip et al. (1978) and Lehn-Jensen (1980a).

Massip et al. (1979) found that after seeding the rate of crystalization from the seeding point to the plugged end of the straw was often different from one straw to another, but Niemann et al. (1981a) observed that seeding could probably be made with more accuracy and in a shorter time by using plastic straws. Massip et al. (1982) observed that automatic seeding is only possible with straws because of the small volume, large surface of contact and thin wall of the straw.

Factors Affecting Temperature Rate Changes and Freeze-Thaw Injury

An obvious factor of freeze-thaw injury appears to be the lowered temperature, but just lowering the temperature does not seem to be detrimental to living cells (Mazur, 1966). The main factor affecting freeze-thaw injury is the osmotic response of the cell. When water freezes it displaces the cryoprotectant suspended in the media, thus resulting in higher concentrations of the cryoprotectant in the unfrozen portion of the media. This increased concentration of solutes destabilizes the cell membrane and allows a net influx of solute into the cell (Mazur, 1977).

Fast cooling rates prohibit adequate osmotic equilibration and dehydration before intracellular freezing occurs. This increases the chances of large, lethal intracellular ice crystals forming at the nucleation temperature. Slow cooling rates allow the cells to adequately dehydrate before small amounts of water can freeze (Mazur, 1977). Leibo (1977b) showed that ovum cooled at 30 C/min are the same size when frozen at -40 C as they were when at 0 C, but ovum cooled at 3.0 C/min are smaller in size when they froze at -40 C. Lehn-Jensen and Greve (1982) and Walter et al. (1975) found a good correlation between functional survival on thawing and the shrinkage of cells at -20 C and

-26 C, respectively. McGann and Farrant (1976) stated that damage following cooling and thawing is mainly due to dilution shock during rewarming, which can be minimized by allowing the cells to shrink at the termination temperature, the last temperature before plunging the sample into liquid nitrogen. Therefore the optimum cooling rate is that rate which is slow enough to prevent formation of intracellular ice and rapid enough to minimize cellular exposure to the high concentration of solutes (Leibo et al., 1970; Mazur, 1980; Maurer, 1978).

The cooling rate for bovine embryos from room temperature to -6.0 C or -7.0 C seems to be immaterial. Bouyssou and Chupin (1982b) brought embryos directly to -7.0 C from room temperature at approximately 5.0 C/min and found no significant decrease in survival. Massip et al. (1982) and Lehn-Jensen (1983) also cooled embryos directly to the nucleation temperature from 20 C and found no adverse effects. Leibo et al. (1974) found that mouse embryos do not survive freezing if cooled at rates of more than 7.0 C/min. Willadsen et al. (1976b) showed that even 1.0 C/min may be too great of a cooling rate for sheep embryos.

The most widely used cooling rates below the nucleation temperature for bovine embryos appear to be between or a combination of 0.1 and 0.3 C/min. (Bilton and Moore, 1977; Renard et al., 1981b; Willadsen et al., 1976a; Elsden et al., 1982; Lehn-Jensen, 1980b; Farrand et al., 1982; Niemann et al., 1982b; Schilling et al., 1980; Smorag et al., 1981a; Trounson et al., 1978b; Wood and Farrant, 1980). Bouyssou and Chupin (1982a,b) transfered bovine embryos at -7.0 C directly to -30.0 C and held them at that temprature for 30 min before plunging them into liquid nitrogen. They found no significant difference between this procedure and a slower, more widely used method.

Since equilibration between internal and external solutions is time and temperature dependent, equilibration requires more time as the temperature is lowered (Mazur, 1966; Leibo and Mazur, 1974; Jackowski, 1977; Mazur and Rajotte, 1981). Therefore a faster cooling rate can be used if the termination temperature is held long enough for osmotic equilibration to occur (Leibo, 1977a), or if the cooling rate is slowed several degrees above the termination temperature, to allow the embryo a longer period of time at the lowered temperature for osmotic equilibration to occur (Willadsen, 1977).

Wood and Farrant (1980) showed that a longer holding time at the termination temperature was not beneficial when the cooling rate was 0.3 C/min. Smorag et al. (1981a) showed, with mouse embryos, that holding the temperature at -40 C for 30 to 45 min was beneficial, but their cryoprotectant was added at 0 C and because of the time temperature relationship, complete equilibration had probably not occured when the termination temperature was reached. Elsden et al. (1982) showed that slowing the cooling rate from 0.3 C/min to 0.1 C/min for the last 3.0 C (-35.0 C to -38.0 C) before plunging into liquid nitrogen was superior to cooling at 0.3 C/min. to -35.0 C and holding that temperature for 30 min before plunging into liquid nitrogen. However, they found no significant difference between cooling at 0.3 C/min to -35.0 C, holding that temperature for 30 min before plunging into liquid nitrogen and cooling to -35.0 C at 0.3 C/min before plunging directly into liquid nitrogen.

Slow cooling rates terminated at a relatively high temperature, so that adequate osmotic equilibration can occur, require fast thawing rates (>360 C/min), whereas slow cooling rates terminated at a

relatively low temperature require slow thawing rates. Studies indicate that cells must be warmed at a slow rate if cooled below -45 C before plunging into liquid nitrogen (Polge and Willadsen, 1978; Willadsen, 1977; Wood et al., 1978; Kasai et al., 1980; Leibo et al., 1974; Bilton and Moore, 1976b; Whittingham and Adams, 1976; Willadsen et al., 1978b). Working with mouse embryos, Wood et al. (1978) and Whittingham et al. (1979) reported adequate survival rates with fast thawing rates from -196 C provided that the slow cooling stage was terminated at relatively high subzero temperatures (-35 C to -45 C) followed by immediate rapid cooling in liquid nitrogen. Lehn-Jensen and Greve (1981), working with bovine embryos, found that the termination temperature could be as high as -20 C and still have acceptable results. Elsden et al. (1982) determined that using a 37 C bath for thawing frozen bovine embryos resulted in higher survival rates than using a 25 C bath when the termination temperature was -35 C or -38 C. Cacherio et al. (1983) found that a 20 C bath resulted in a higher percentage of embryos developing than a 37 C bath. Whereas, Massip et al. (1982) found no difference. When the termination temperature is relatively high a thaw bath at 20 C (Lehn-Jensen, 1980a), 25 C (Tervit and Elsden, 1981; Trounson et al., 1978a; Willadsen et al., 1978a; Massip et al., 1979; Farrand et al., 1982), 35 C (Bouyssou and Chupin, 1982a), or 37 C (Lehn-Jensen and Greve, 1981; Renard et al., 1981b; Bouyssou and Chupin, 1982b) yields acceptable results.

Factors Affecting Nucleation and Nucleation Temperatures

Nucleation is the formation of ice crystals. The optimum nucleation temperature for bovine embryos seems to be similar for sheep

(Bilton and Moore, 1976a; Willadsen et al., 1976b), mouse (Whittingham, 1977; Kasai et al., 1980) and rat (Miyamoto and Ishabashi, 1978) embryos. Successful results have been reported for bovine embryos at nucleation temperatures of -3 C with 1.5M DMSO and 1.0M glycerol (Bilton and Moore, 1977; Bilton, 1980), -6 C with DMSO and glycerol (Lehn-Jensen, 1980a), and -7 C with 1.5M ethylene glycol, 1.33M glycerol and 0.625M glycerol plus 0.625M DMSO (Elsden et al., 1982), 1.5M DMSO (Farrand et al., 1982; Lehn-Jensen, 1980b), and 1.5M DMSO and 1.4M glycerol (Bouyssou and Chupin, 1982b), and 1.0M glycerol (Niemann et al., 1982b). Trounson et al. (1978b), using 1.5M DMSO, found no difference between -5.5 C, -6.0 C and -7.0 C as the nucleation temperatures.

Nucleation can be accomplished at or below the nucleation temperature by the introduction of an ice crystal of frozen media into the media to be nucleated (Smorag et al., 1981a; Trounson et al., 1978b; Wilmut and Rowson, 1973), by using the Peltier effect (Christmas, 1983), by a cold liquid nitrogen vapor stream blasted on the sample tube (Massip et al., 1982; Kanagwa et al., 1979), or by clamping forceps supercooled in liquid nitrogen to the wall of the vessel containing the embryos (Niemann et al., 1982b; Bouyssou and Chupin, 1982b; Bilton, 1980). Trounson et al. (1978b) found no difference in survival rates when they compared using a frozen crystal of media and cold forceps to induce crystallization.

Supercooling is the cooling below the nucleation temperature without the formation of ice crystals. Whittingham (1977) noted that a medium containing 1.5M DMSO that was not seeded may supercool to -21.0 C before ice spontaneously forms. Excessive supercooling has been shown

to reduce the survivability of embryos of bovine and mice (Bilton and Moore, 1976a; Moore and Bilton, 1977; Whittingham, 1977). Ice crystal formation causes a release of latent heat of fusion. This release of latent heat causes an increase in the cooling rate until the sample being cooled reequilibrates with the temperature of the cooling bath (see figure 1). Whittingham (1977) and Leibo (1977a) determined that this increased cooling rate, after the latent heat release, was the lethal agent, not just the process of supercooling. Maurer (1978) stated that the induction of nucleation at a relatively high temperature reduced the subsequent rise in temperature, caused by the release of latent heat during the phase change from liquid to solid, kept the cooling rate from dramatically increasing and prevented excessive supercooling.

Maurer (1978) stated that for embryo survival the freezing medium must be nucleated 1 or 2 degrees below the medium's freezing point. This necessity was clearly illustrated with sheep embryos (Moore and Bilton, 1977). In this study 5 treatments (not seeded, seeded at -2.5 C, -5.0 C, -7.5 C and -10.0 C) were used. No developmental differences were found between the embryos seeded at -2.5 C, -5.0 C and -7.5 C. Conversely, the embryos seeded at -10.0 C and not seeded failed to show any signs of development.

Factors Affecting Cryoprotective Agents and Freeze-Thaw Injury

The requirements for an effective cryoprotective agent are threefold: 1. it must be nontoxic to the tissue that it is affording protection, 2. it must have the ability to permeate the cell membranes, and 3. it must have the capacity to dissolve electrolytes (Douzou, 1977).

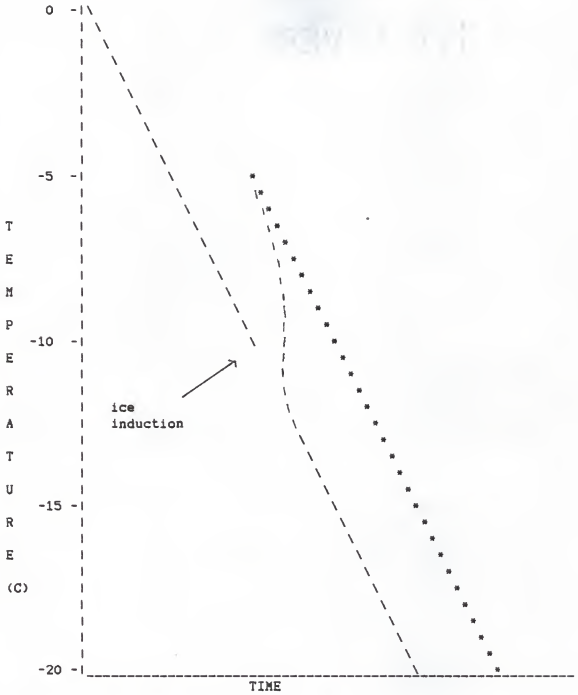

Figure 1. Latent Heat Release After Supercooling.

Diagrammatic illustration of a typical cooling curve showing the elevation of temperature resulting from the introduction of ice formation at -10.0 C in a sample cooling at 0.5 C/min and the increased velocity of cooling (>3 C/min) as the sample reequilibrates with the temperature of the cooling bath, which is cooling at 0.5 C/min. Starred line represents cooling rate from elevated temperature if samples are transferred to another bath cooling at the rate of 0.5 C/min from the higher temperature (Whittingham, 1977).

Cryoprotectants. The two most widely used and most effective cryoprotective agents used for bovine embryos are dimethlysulfoxide (DMSO) (Bilton and Moore, 1977; Bouyssou and Chupin, 1982b; Lehn-Jensen and Greve, 1978, 1980; Renard et al., 1981b; Trounson et al., 1978a; Willadsen et al., 1976a) and glycerol (Bilton and Moore, 1979; Bouyssou and Chupin, 1982b; Lehn-Jensen et al., 1981; Niemann et al., 1981a; Smorag et al., 1981a).

Lehn-Jensen (1981) found that DMSO, in concentrations that yielded cryopreservation, created greater osmotic and pH stress than did glycerol. Lehn-Jensen and Greve (1981) concluded that in the present way of application glycerol is a better cryoprotectant than DMSO.

Bilton and Moore (1979) found that 1.0M glycerol was equal to 1.5M DMSO. Bouyssou and Chupin (1982b) found that 1.4M glycerol was better than 1.5M DMSO after 6 and 24 h in culture post-thaw, but that after 48 h in culture there was no significant difference. Smorag et al. (1981a) had results indicating that 3.0M ethylene glycol is superior to 3.0M DMSO as a cryoprotectant. Elsden et al. (1982) found that 1.33M glycerol was equal to 0.625M glycerol plus 0.625M DMSO, with a 33% and 32% survival rate, respectively, and they were both superior to 1.5M ethylene glycol. Renard et al. (1981a) found a seldom used cryoprotectant, 1.2M propandiol, equally effective as DMSO or glycerol.

Renard et al. (1981b) reported pregnancy rates as high as 73% using DMSO and selected frozen-thawed bovine embryos. Using 1.5M DMSO Bilton and Moore (1976b) had a pregnancy rate of 23% from 39 frozen-thawed bovine embryos, while Massip et al. (1979) had 24% from 89 embryos, Schneider and Hahn (1979) had 36% from 19 embryos and Schneider et al. (1980) had 34% from 68 embryos. Also using 1.5M DMSO Willadsen et al.

(1978a) had a survival rate post thawing of 64% from 76 bovine embryos and Trounson et al. (1978a) had 61% from 64 embryos frozen. Lehn-Jensen (1981) used 1.4M glycerol and achieved a 64% pregnancy rate on 53 frozen-thawed bovine embryos. Lehn-Jensen and Greve (1982), using 1.4M glycerol, reported 66% survival rate from 123 frozen-thawed bovine embryos. Bouyssou and Chupin (1982a) used 0.4M, 0.9M and 1.4M glycerol and achieved 88% good embryos at thawing.

Wilmut and Rowson (1973) tried 0.3M sucrose as a cryoprotectant for bovine embryos and Wilmut (1972) tried 0.6M sucrose as a cryoprotectant for rabbit embryos, but neither reported any survival after thawing.

Toxicity. Meryman (1971) reported that with DMSO, at concentrations greater than 4.5M, the human red cell electrolyte distribution was altered significantly enough to cause toxicity in the cell, but that with glycerol, at concentrations up to 10M, there was no significant change in the electrolyte distribution. Since the molarity of the cryoprotectants used in freezing bovine embryos is well below these toxic levels it can be assumed that these compounds are nontoxic and safe.

Permeation. The requirement of permeation is related to the type of tissue frozen. Mazur and Rajotte (1981) showed that a ^{14}C labeled 2.0M glycerol solution did not internally equilibrate with fetal rat pancreases at 0 C, even after 180 min, but that ^{14}C labeled 1.0M and 2.0M DMSO did equilibrate after only 20 min. It appears that fetal rat pancreases require the permeation because the survival rates were significantly lower with glycerol. However, Mazur (1980) reported that permeation is not necessary for the survival of frozen-thawed red blood cells. Permeation is apparently necessary for the survival of frozen-

thawed embryos. Both DMSO and glycerol have been found to permeate bovine embryonic cells (Leibo, 1977b; Jackowski and Leibo, 1976; Leibo et al., 1974; Jackowski, 1977).

When cryoprotectants are added, generally they must be added in a step-wise fashion to avoid an abrupt osmotic change that may damage a cell's membrane. Miyamoto and Ishabashi (1978) concluded that it was necessary to add the cryoprotectant in 3-steps, with a 10 min equilibration period for each step. Leibo et al. (1974) concluded, with mouse embryos and DMSO, that the sequence of osmotic volume changes associated with dehydration and rehydration of the embryos is not harmful to the cell's membranes. Wilmut (1972) found that the optimum addition of DMSO was in increments of 0.5M to 1.0M at 5 to 10 min intervals. Lehn-Jensen and Greve (1981) added 1.4M glycerol in 2-steps (0.0M - 0.7M - 1.4M) with apparent success and Lehn-Jensen (1983) successfully added 1.4M glycerol in 1-step. Willadsen et al. (1978b), Leibo et al. (1974), Maurer et al. (1977), Cacherio et al. (1983) and Whittingham (1975) found that DMSO can be added in just 1-step with no apparent damage.

Cryoprotectants provide protection during the freeze-thaw process because of their colligative properties. These colligative properties initially cause the cell membranes to leak water, osmotically balancing the internal and external cellular concentrations, thereby dehydrating the embryo. But the cryoprotectant, which permeates cells hundreds to thousands of times slower than water (Mazur, 1977), concurrently leaks into the cell carrying water along with it and causing the cell to reexpand. Leibo (1977b) demonstrated, in a series of photographs of mouse embryos, how the cell membranes collapsed when a cryoprotectant

was added and then slightly reexpanded, but not to their origional volume. The reason for this reexpansion is that the dehydration of the embryo occurs faster than the diffusion of the cryoprotectants into the cell. Thus the embryo begins to dehydrate and shrink, then simple diffusion of the cryoprotectant through the cell membranes causes reexpansion of the cell because of the water drawn along with the cryoprotective agent (see figure 2). The concentration of cryoprotectant required is therefore low enough to be nontoxic and prevent solution effect injury, but high enough to adequately dehydrate the embryo to prevent intracellular ice formation.

Thermal Shock. The stresses of thermal shock damage cell membranes. Daw et al. (1973) demonstrated that ^{14}C labeled sucrose, a normally nonpermeating compound to the red cell, was found in the red cells after freeze-thawing. Mohr and Trounson (1981) showed that freeze-thawing bovine embryos can result in a break down of the junctional complex. The juctional complex includes the desmosomes, which serve a strong intercellular bonds or "spot welds" that hold the trophoblast cells together. Without these bonds the hydrostatic pressure accompanying the blastocele expansion can not build up, because the membranes could not hold the pressure and would leak water (Ducibella, 1977). Therefore, break down of the desmosomes, which indicates structural damage, can be indirectly observed by the collapse of the blastocelic cavity.

Cryoprotectants are needed to minimize thermal shock damage. Lehn-Jensen (1981) observed that embryos frozen in glycerol appear to maintain their normal morphology better than embryos frozen in DMSO. Smorag et al. (1981a) observed that 3.0M glycerol gave the highest

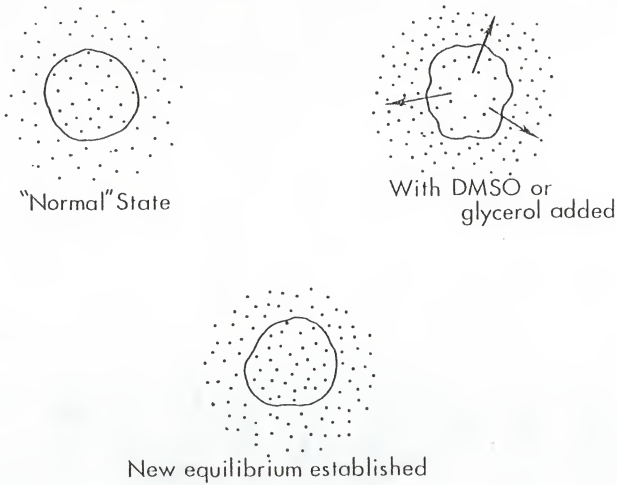

Figure 2. Cellular Dehydration

When the concentration of molecules such as DMSO or glycerol suddenly increases, water initially leaves the cell because of its increased permeability. The DMSO or glycerol does, however, penetrate into the cell, but at a much slower rate, causing a reexpansion of the cell.

percentage of morphologically normal and normal developing embryos as compared with DMSO, DMSO plus ethylene glycol, DMSO plus glycerol and ethylene glycol plus glycerol.

Massey et al. (1982) and Hoppe and Bavister (1983) found that the zona pellucida is unnecessary for the implantation of fresh blastocysts. Wilmut and Rowson (1973) and Tervit et al. (1981) both had results suggesting that an intact zona pellucida is highly beneficial to the survival of frozen-thawed bovine embryos. Bouyssou and Chupin (1982b) found that the zona pellucidas of frozen-thawed bovine embryos were more severely cracked with DMSO, but that smaller cracks were observed more frequently with glycerol. However, a cracking of the zona pellucida does not indicate cell death. Willadsen et al. (1978b) found that 3 of 4 frozen-thawed bovine embryos with a cracked zona pellucida developed normally, while 5 of 7 frozen-thawed bovine embryos without a cracked zona pellucida developed normally.

Cryoprotectant Removal. Cryoprotectant removal appears to be an important step for embryo survival. Whittingham (1974) reported that the removal of DMSO from blastocysts soon after thawing is clearly essential for good survival, probably because this may reduce any toxic effects and osmotic damage. Willadsen et al. (1978b) noted that the removal of DMSO before transfer was necessary for good survival and obtained 1 pregnancy from 20 frozen-thawed bovine embryos (5%) that had not had the DMSO removed, compared to 9 pregnancies from 16 frozen-thawed bovine embryos (56%) that had the DMSO removed. Lehn-Jensen (1980b) transfered 4 fresh embryos in just a Phosphate Buffered Saline (PBS) solution and 4 fresh embryos in a 0.6M glycerol solution and obtained no pregnancies from the glycerol and 2 from the PBS.

Tsunoda and Sugie (1977a), Kasai et al. (1980) and Bank and Maurer (1974) all concluded that cryoprotectant removal resulted in higher survival rates if it was removed at 37 C versus 0 C. Whittingham (1974) found that removal at 37 C was better than removal at 20 C. Leibo (1976) reported a similar pattern in which he put bovine red cells into concentrated glycerol solutions, solutions which were equal to the concentration changes encountered during freezing, and found that the membrane damage was greater when these changes were carried out at -5 C and 10 C rather than at 20 C.

Embryo Rehydration. Rehydrating frozen-thawed embryos requires a lower osmotic gradient change than used for initial dehydration of the embryo because freeze-thawing of bovine embryos can cause loss of integrity of the plasma membrane (Mohr and Trounson, 1981). Cellular rehydration and cryoprotectant removal is generally a 6-step process (1.4M, 1.1M, 0.93M, 0.70M, 0.47M, 0.23M, 0.00M) designed to maintain cellular integrity. This procedure is time consuming and may result in accidental loss or misidentification of the embryos.

Efforts have been made to reduce the number of equilibration steps without reducing survivability so that it is a more efficient process. In 1979 Kanagwa et al. attempted to puncture the zona pellucida and then remove the DMSO in 1-step, but only 1 calf was born from 23 embryos transfered (4%). In 1980 Kasai et al. reported that 2.0M DMSO could be removed in a 3-step process. From the 2.0M DMSO in the freezing media they transfered the embryo to a solution that contained 2.0M DMSO plus 0.5M sucrose. After a 10 min equilibration period the embryo was transfered to a solution containing only 0.5M sucrose. The embryo was then transfered to a solution that contained no sucrose or DMSO. Their

results with mice showed that, although not significantly different, this procedure resulted in a higher percentage of mouse embryos developing into blastocysts than did their usual 3-step procedure. In 1981(a) Renard et al., using frozen-thawed bovine embryos frozen in 1.2M propandiol or 1.5M DMSO, found this same sucrose gradient beneficial. Also in 1981a, Niemann et al. found the sucrose gradient beneficial with 1.0M glycerol. Niemann et al. (1982b) used 1.4M glycerol to freeze bovine embryos and found that the sucrose gradient was significantly better than their usual 5-step removal procedure (83% versus 53%).

In 1982 Renard et al. demonstrated a 1-step process for the thawing and rehydration of frozen cattle embryos. The embryos were frozen in .5ml French straws which contained three solutions seperated by air bubbles. The first solution (starting at the plunger) was a nutrient medium, Milieu B2 (Menezo, 1976), followed by a large air bubble (90% N_2, 5%O_2, 5% CO_2). The middle solution, containing the embryo, was composed of PBS plus 20% fetal calf serum and 1.5M glycerol. A small air bubble seperated this solution and the last solution, which comprised half the volume of the straw. The last solution was PBS plus 20% fetal calf serum plus 0.25M sucrose. All straws were sealed with a plastic plug (see figure 3). The second air bubble was small enough that after thawing, and the straw was turned on end and tapped, the small bubble would rise to the end of the straw, thus mixing the sucrose and glycerol solutions. Twenty-two embryos were thawed and rehydrated with this technique. The total contents of the straws were then non-surgically transfered to recipient cows. At d 70 of gestation 11 (50%) were diagnosed as pregnant. Leibo (1983) used a similar type .25ml straw with similar compartments and achieved 36% pregnancies (115 total

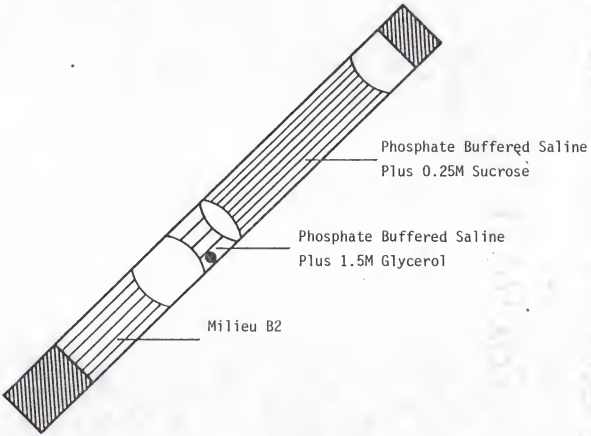

Figure 3. 1-Step Straw (Renard et al., 1982)

transfers).

Timed addition of a rehydrating solution also appears to be an effective process which reduces embryo handling. Whittingham et al. (1972) demonstrated a timed interval titration process for cryoprotectant removal. PBS solutions containing no DMSO were added, in 5 min intervals, to 0.1ml of a 1.6M DMSO solution. Through the use of this timed interval titration process it became unnecessary to move the embryos and thus avoid the chance of misidentification or loss of the embryo. Bilton and Moore (1977) sucessfully used the same process with 1.0M glycerol. Bank and Moore (1974) also developed a timed interval titration process. Upon thawing of rabbit embryos frozen in 1.0M DMSO they added a PBS solution that was four 4 the initial osmotic strength of the PBS. This doubled the total solution volume, changed the total mOsm by less than 300 mOsm, and most importantly diluted the DMSO quickly. They continued with 3 more steps similar to the first one. On the fifth addition they used a PBS solution that was only 1/2 the osmolarity of the original stock solution of PBS. This last step completely removed the DMSO and changed the osmolarity only 178 mOsm. Schneider and Hahn (1979) used this same concept and found that this slightly hypertonic treatment resulted in a slower water uptake by the embryos during the dilution process and prohibited excessive swelling of the blastomeres as compared to a normal 6-step removal procedure.

Other Components That Indirectly Aid In Cryopreservation

Bovine serum has been shown to be an essential agent for the development of embryos in culture (Kane, 1978). Tsunoda and Sugie (1977b) showed that a significantly higher proportion of rabbit eggs frozen and thawed in Ringers or PBS with added serum developed into

blastocysts than in the absence of serum. Allen et al. (1982) showed that 10% serum tended to be, but not significantly, better than 5% serum. However, Lehn-Jensen (1980a), Massip et al. (1979), Tervit and Elsden (1981) and Trounson et al. (1976b, 1978a) all used 20% serum.

The component or components in serum that are necessary for development are unknown, although Whittingham (1976) has speculated that development would not occur without serum because there would be a lack of bicarbonate ions. Wright et al. (1976) showed that heat treated fetal calf serum caused bovine blastocysts to expand and hatch more readily than bovine serum albumin. Voelkel et al. (1981) showed that heat treated newborn calf serum resulted in significantly less bacterial growth than heat treated or non-heat treated steer serum or lamb serum. Allen et al. (1982) theorized that since serum from young animals appears to promote growth and embryonic development better than serum from adults, there must be a compound or compounds present in the serum of adults that promotes growth, or a compound or compounds absent in the serum of young animals that is present in the serum of adults that retards development.

Since the addition of blastokinin or serum to culture media enables rabbit, mouse, bovine and rat morula to blastulate, it has been concluded that serum contains blastokinin or a blastokinin-like compound that aids blastulation (Gulyas and Krishman, 1971). Therefore, the component in serum that promotes blastulation must be present in the serum of the young animal and not be present in the mature animal, or the mature animal has another component which blocks blastulation.

Serum does have some properties that provide some known protection. It contains lipoproteins which form a layer in the surface of the

culture dish which cells must circumvent in order to attach themselves to the dish (Temin et al., 1972; Farrand, 1980). Therefore with serum added the embryo cannot attach to any surface. Serum can also repair membrane damage caused by the stresses of freezing with its lipid and fatty acid components (Edidin, 1977).

Holding Medium. The most widely used holding medium is Phosphate Buffered Saline (PBS). It enables the pH, osmolarity and ingredients to be controlled (Whittingham, 1976). Trounson et al. (1976a) showed that PBS had a more stable pH than TCM 199, which caused considerable degeneration and retardation of egg development, although, sheep embryos have developed at a pH as low as 6.8 (Moore, 1982) and mouse embryos have developed from 2-cell to the blastocyst stage at a pH range of 5.87 to 7.78 (Brinster, 1965). Prather and Spire (1984) showed that pH is not a critical factor when embryos are in a frozen state at -196 C.

Kuzan and Wright (1981) showed that Hams F-10 supported morula development better than minimal essential medium, although both expanded blastocysts. They also showed that minimal essential medium was superior for hatching blastocysts from the zona pellucida. Tervit and Elsden (1981) showed that embryos in PBS plus fetal calf serum developed more readily than those in Hams F-10 after a 24 h culture period.

Antibiotics. Antibiotics are added to prevent bacterial growth. Hafez (1971) stated that acetosulphamine and sulfamerizine tended to inhibit embryonic development and that streptomycin at concentrations greater than 1.0% inhibited cleavage. He also stated that survival at low temperatures is not affected by 7.5mg/ml streptomycin, 4.0mg/ml chloromycedin, 6.5mg/ml paromycin or 23.9mg/ml penicillin.

EXPERIMENTAL PROCEDURE

A total of 228 bovine embryos were used to evaluate different methods of cryopreservation. These embryos were collected during a 15 mo period from Hereford, Simmental and Angus cows at Kansas State University and from privately owned Hereford and Holstein cows. Cows were synchronized with $PGF_{2\alpha}$[1] or an analogue[2] and superovulated with 8 or 10 injections of FSH[3] (Appendix Table 1). Observed estrus detection was aided by androgenized cows fitted with chinball markers and Kamar[4] mount detectors. The cows were bred at standing heat and 12 h or at 12 and 24 h after the onset of estrus using 2 straws of frozen semen at each breeding. The embryos were collected from the cows either 7 or 8 d following first observed estrus.

Cows to be collected were given an epidural injection of .5 - .75ml 2% Lidocaine[5] per 45.4Kg of body weight, and their external genitalia were throughly scrubbed with an iodine base disinfectant scrub[6]. A 2- or 3-way (16, 18, 20 or 24 French) Foley catheter[7] was inserted through the cervix. The cuff was inflated with 6 to 12ml of flushing media so that its final position was located just anterior to the anterior cervical ring. A reducing adapter, fitted with a latex tube was connected to the 2-way Foley. Sixty milliter syringes were then used to

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[1]Lutalyse®, The Upjohn Company, Kalamazoo, Michigan.
[2]Estrumate®, Bayvet Division, Cutter Laboratories, Inc., Shawnee, Kansas.
[3]FSH-P. Burns-Biotec Laboratories, Inc., Omaha, Nebraska.
[4]Kamar Inc., Steamboat Springs, Colorado.
[5]2% Lidocaine, Elkins-Sinn, Inc., Cherry Hill, N.J.
[6]Betadine surgical scrub. The Purdue Frederick Company, Norwalk, Connecticut.
[7]Travenol Laboratories, Inc., Deerfield, Illinois.

deliver and recover fluid from the uterine horns (Appendix Figure 1). Forty to 80ml of flushing medium (Appendix Table 2) were injected into either horn of the uterus, manipulated, and aspirated back as described by Ramerez-Godinez et al. (1980). The syringes were then emptied into settling flasks or 120ml flat bottom bowls[8]. The bowls were searched directly while aliquots were drawn off the bottom of the flasks for examination. When using the 3-way catheters the fluid was returned directly to the flat bottomed bowls.

An inverted microscope (40X, 100X, 200X) was used for all fluid scanning, embryo evaluation and embryo photography. When an embryo was located it was transfered to a small culture dish containing Short Term Culture Medium (STCM) (Appendix Table 3.) The culture dish was placed on a slide warmer at 37 C until the start of the dehydration procedure.

After all the embryos from University owned cattle were located they were assigned to 1 of 24 treatment groups (Table 1).

The dehydration procedure with 1.4M glycerol[9] or 1.5M DMSO[10] was then initiated. The dehydration procedure was a 3-Step or a Timed Interval Titration (TIT) procedure, both were carried out at 37 C.

The embryos were dehydrated in glycerol or DMSO by a 3-Step process (glycerol, 0.00M, to 0.47M, to 0.93M and to 1.4M; DMSO, 0.00M, to 0.50M, to 1.00M and to 1.5M). A 10 min equilibration period was allowed for each step (Appendix Table 4).

The TIT procedure was accomplished by placing the embryos in 5ml of STCM in a 15ml culture dish[11] and adding the necessary amounts of

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[8]Ova-Trans. Veterinary Concepts, Spring Valley, Wisconsin.
[9]Glycerol. American Scientific Products, McGaw Park, Illinois.
[10]Dimethylsulfoxide, 99.5%. T.P.Marketing Co., San Antonio, Texas.
[11]Falcon 3002. Divison Becton, Dickenson and Co., Oxnard, California.

TABLE 1. EXPERIMENTAL DESIGN[1]

		6-Step Rehydration		Sucrose Rehydration		1-Step Rehydration	
Thaw Temperature		27	37	27	37	27	37
DMSO	3-Step Dehydration	T11	T12	T13	T14	T15	T16
	T.I.T.	T21	T22	T23	T24	T25	T26
Glycerol	3-Step Dehydration	T31	T32	T33	T34	T35	T36
	T.I.T.	T41	T42	T43	T44	T45	T46

[1]Figure 4.

concentrated dehydration medium (Appendix Table 5) with small syringes. This medium was added so that a circular swirling action was created in the dish.

The embryos dehydrated in glycerol by the TIT process were placed in 5.0ml of STCM, and 1.4ml, 2.5ml and 6.1ml of a 2.10M solution of glycerol was titrated into the STCM in intervals of 10 min. This changed the concentration of glycerol from 0.00M, to 0.47M, to 0.93M and to 1.4M, thus simulating the 3-step process described earlier. The embryos dehydrated in DMSO by the TIT process were treated in a similar fashion. This process added 1.4ml, 2.6ml, and 6.0ml of a 2.25M DMSO solution (Appendix Table 6).

After complete dehydration the embryos were stage (late morula, early blastocyst, blastocyst, expanded blastocyst, hatched blastocyst)

and quality graded (Table 2), then loaded into 1/4 or 1/2ml plastic straws[12] (Appendix Figures 2 and 3). The 1/4ml straws contained only freezing medium and were sealed with a pair of hemostats. The 1/2ml straws were loaded with 3 seperate fluids; STCM, freezing medium containing the embryo, and a 0.5M sucrose[13] solution in PBS, and were plugged with a plastic plug[14] similarly described by Renard et al. (1982).

The loaded straws were placed in an automatic freezer[15] set at 20 C. All straws were then placed near the liquid nitrogen (LNG) vent and were in direct contact with a hemostat situated in the path of the LNG exiting the vent. After an equilibration period of 5 min at 20 C the embryos were cooled to -6.0 C at 1.0 C/min, at which time a blast of LNG induced seeding similar to that of Massip et al. (1982) and Kanagwa et al. (1979). The embryos were then cooled to -30 C at 0.3 C/min and then to a termination temperature of -38 C at 0.1 C/min (Appendix Figure 4). At the termination temperature the straws were removed and plunged into LNG at -196 C. Storage of embryos in LNG ranged from 1 to 352 d.

Following storage the embryos were thawed in a water bath at 27 C or 37 C. The straws were immersed and gently agitated for a period of 10 s after complete decrystallization. After thawing the embryos were again quality graded (Table 2). The embryos frozen in 1/4ml straws were rehydrated by one of two ways, both at 37 C, either a 6- or 3-Step procedure. The 1/2ml straws were rehydrated by a 1-Step procedure.

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[12]Straws, Paillette fine cristal (1/2), Mini paillette cristal courte (1/4). I.M.V., Minneapolis, Minnesota.

[13]Sucrose. MCB Manufacturing Chemists Incorporated, Cincinnati, Ohio.

[14]Plastic Plugs. Continental Plastics, Delavan, Wisconsin.

[15]Cryo-Med. model 1000. Mt. Clemens, Michigan.

TABLE 2. EMBRYO QUALITY CLASSIFICATIONS

Prefreezing Quality Grade (1-3)	Postfreezing Quality Grade (1-5)	Description[1]
1	1	Excellent, no visible imperfections.
2	2	Good, Trivial imperfections, few extruded blastomeres, slightly asymmetrical.
3	3	Fair, Small amounts of degeneration, slightly developmentally retarded.
	4	Poor, Retarded 1-2 days, extruded blastomeres, asymmetric shape.
	5	Degenerate, Retarded >2 days, little cellular integrity.

[1]Appendix Figure 6.

The 6-Step rehydration procedure consisted of moving the embryos through a series of dishes that have equally reduced concentrations of cryoprotectant, while allowing a 10 min equilibration period for each step. The concentration of DMSO was changed from the initial freezing medium of 1.50M, to 1.25M, to 1.00M, to 0.75M, to 0.50M, to 0.25M, to 0.00M and glycerol from the initial freezing medium of 1.40M, to 1.17M, to 0.93M, to 0.70M, to 0.47M, to 0.23M, to 0.00M (Appendix Table 8).

The 3-Step procedure previously described by Kasai et al. (1980), Renard et al. (1981a) and Niemann et al. (1982b). The embryo was moved from the freezing medium to a dish that contained the initial freezing medium (1.4M glycerol or 1.5M DMSO) plus 0.50M sucrose. At 10 min intervals the embryo was moved to a 0.50M sucrose solution, then to STCM

(Appendix Table 9, 10).

The 1-Step sucrose rehydration process for the 1/2ml straws was similar to the 3-Step sucrose rehydration, but was accomplished within the straw itself. After thawing, the straw was held on end and tapped lightly until the small air bubble rose to the plastic plug (Appendix Figure 5). This mixed the embryo, in the glycerol or DMSO, with the sucrose and thus rehydrated the embryo while allowing for the sucrose protection. The straw was the held in this position for 10 min before it was transfered to STCM.

All embryos were quality graded (Table 2) 10 min after rehydration. Following grading, 104 of the 228 embryos were cultured in STCM at 37 C with final viability assessment based on in vitro development after 12 h of culture. One-hundred seven embryos initially frozen were transfered to recipient cows, 13 were lost due to explosion of the straw upon thawing, and 4 were outside the stage of development range early blastocyst to expanded blastocyst. These 124 embryos were eliminated from the final analysis, however, their intermediate quality grades, where available are included in this report. An overview of the experimental procedure is illustrated in figure 4.

Culture and morphological examination appears to be an adequate method of assessing viability (Bilton and Moore, 1979; Linares and King, 1980). Niemann et al. (1981b) has estimated only an 8-10% increase in accuracy of evaluation by using more sophisticated methods such as fluorescent dyes.

All data were analyzed by analysis of variance using the General Linear Models procedure of Statistical Analysis Systems (SAS, 1983).

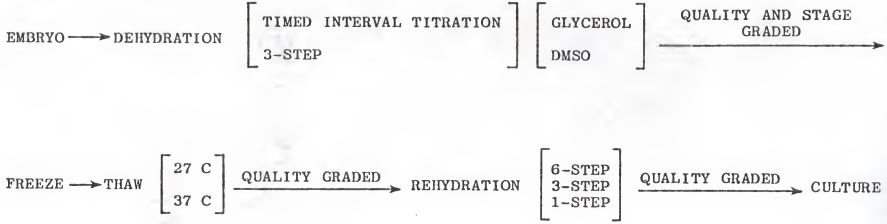

Figure 4. Experimental Procedure

RESULTS AND DISCUSSION

Factors Affecting In Vitro Survival

In vitro culture of post-thawed bovine embryos was affected only by the initial Quality Grade (Q.G.) of the embryo. Quality grade 1 and 2 embryos survived significantly more often than Q.G. 3 embryos (64.8%, 67.8% vs. 29.1%). This finding agrees with Shea et al. (1983) and Kennedy et al. (1983), who found better quality embryos have a higher chance of surviving the freeze-thaw process (Table 3a).

In this study a comparison of 1.4M glycerol and 1.5M DMSO as the cryoprotectants showed no significant difference in survival rates (Table 3a). Bilton and Moore (1979) also found no difference when they compared 1.0M glycerol to 1.5M DMSO. Bouyssou and Chupin (1982b) found that 1.4M glycerol was a better cryoprotectant than 1.5M DMSO.

When the moSm of the freezing solutions were calculated it was found that the actual moSm was similar for 1.4M glycerol (1663 calculated vs. 1676 actual), but that the actual value for 1.5M DMSO was not as close to the calculated value (1755 calculated vs. 1980 actual). This variation may be explained by the fact that the DMSO used in this experiment was only 99.5% pure and may have contained some components that greatly altered the final concentration. This higher osmolarity would result in the embryos being more preserved (Smorag et al., 1981b).

The Timed Interval Titration (TIT) dehydration procedure was not different (P=.59) from the the 3-step procedure (57.2% vs. 50.6% survival) as shown in Table 3a. This supports the use of the TIT dehydration procedure since it reduces the amount of handling of the embryo that is required during the dehydration procedures.

TABLE 3a. LEAST SQUARES MEANS FOR PERCENTAGE EMBRYO SURVIVAL BASED ON SINGLE FACTORS

Treatment	(n)	Survival (%)
Quality Grade		
1	65	64.8[a]
2	26	67.8[a]
3	13	29.1[b]
Cryoprotectant		
DMSO	51	49.1
glycerol	53	58.7
Dehydration Procedure		
Timed Interval Titration	50	57.2
3-Step	54	50.6
Thaw Temperature		
27 C	55	50.3
37 C	49	57.5
Rehydration Procedure		
6-Step	37	65.7
3-Step (sucrose)	29	52.4
1-Step (sucrose)	38	43.5

[a, b]Means in the same paragraph with different superscripts are different (P<.05).

The thaw temperatures (27 C vs 37 C) were not found to be significantly different (Table 3a). Massip et al. (1982) had similar results, whereas, Cacherio et al. (1983) found that 20 C resulted in higher percentage survival than 37 C. However, a direct comparison of these studies to the present study is not possible since information on the length of time for temperature equilibrium after decrystalization is not available.

No significant differences were found in embryo survival between the rehydration procedures (Table 3a). However, the 6-Step tended to

yield higher survival rates than the 1-Step (P=.08). It has been previously reported that the sucrose gradient (3-Step) resulted in higher survival rates than the 6-Step rehydration procedure (Renard et al., 1981a; Niemann et al., 1981a, 1982b). While Kasai et al. (1980) found no significant difference. Previous studies showing comparisons between the 3- or 6-Step rehydration procedures with the 1-Step rehydration procedure have not been made.

The length of embryo storage had no effect on survival in this study, this agrees with Lyon et al. (1981) who also found no significant difference between length of storage and survival after thawing. In their studys mouse embryos were frozen up to 5 y with no decrease in survival rates. The superovulation regime was not included in the final model of the present study because superovulation with gonadotropins has been found to have no effect on development or freeze-thaw survival of embryos (Smorag et al., 1981b; Gates, 1956). Maurer (1978), stated that there may be a donor effect, however no donor or sire effect were found in this study.

The stage of development by Quality Grade interaction (Table 3b) indicates that higher quality and more advanced embryos survive freeze-thawing more often than lower quality, less developed embryos (P<.05). The advance of embryos through developmental stages indicates a high degree of inherent viability. Developmentally advanced embryos would then have a lesser chance of containing cellular degeneration which would impair freeze-thaw survivability.

The rehydration procedure by thaw temperature interaction (Table 3b) indicates that the 3-Step rehydration procedure resulted in higher survival rates if the embryos were first thawed at 27 C (P<.05). The

TABLE 3b. LEAST SQUARES MEANS FOR PERCENT EMBRYO SURVIVAL BASED ON TWO FACTOR INTERACTIONS

Interaction	(n)	Survival (%)
Stage of Development X Quality Grade		
Expanded Blastocyst X Quality Grade 1	51	62.5^{ab}
Expanded Blastocyst X Quality Grade 2	9	51.8^{abc}
Expanded Blastocyst X Quality Grade 3	3	73.3^{ab}
Blastocyst X Quality Grade 1	6	70.6^{ab}
Blastocyst x Quality Grade 2	4	86.2^{a}
Blastocyst X Quality Grade 3	3	7.5^{bc}
Early Blastocyst X Quality Grade 1	8	61.3^{ab}
Early Blastocyst X Quality Grade 2	13	65.4^{ab}
Early Blastocyst X Quality Grade 3	6	6.4^{c}
Rehydration Procedure X Thaw Temperature		
6-Step X 37 C	16	82.4^{a}
6-Step X 27 C	21	49.1^{ab}
3-Step X 37 C	14	33.3^{b}
3-Step X 27 C	15	71.6^{a}
1-Step X 37 C	20	56.7^{ab}
1-Step X 27 C	19	30.2^{b}
Cryoprotectant X Rehydration Procedure		
Glycerol X 6-Step	17	83.8^{a}
Glycerol X 3-Step	14	52.4^{ab}
Glycerol X 1-Step	22	39.9^{b}
DMSO X 6-Step	20	47.7^{b}
DMSO X 3-Step	15	52.5^{ab}
DMSO X 1-Step	16	47.1^{b}
Dehydration Procedure X Quality Grade		
Timed Interval Titration X Q.G. 1	36	57.4^{ab}
Timed Interval Titration X Q.G. 2	7	101.9^{a}
Timed Interval Titration X Q.G. 3	6	12.4^{c}
3-Step X Q.G. 1	29	72.1^{a}
3-Step X Q.G. 2	19	33.8^{bc}
3-Step X Q.G. 3	7	45.7^{ab}

a, b, cMeans in the same interaction with different superscripts are different (P<.05).

thaw bath temperatures were not found to significantly influence the survivability of embryos rehydrated by the 6- or 1-Step procedure.

The cryoprotectant by rehydration procedure interaction indicates that neither cryoprotectant works well with the 1-Step rehydration procedure (Table 3b). In this study the osmotic changes encountered in the 1-Step straws may be of a magnitude that the sucrose may not provide adequate protection from these osmotic gradients. The higher osmolarity of the DMSO freezing solution used in this study makes this osmotic change even greater. The reduction in embryo survival seen in the 1-Step method was not seen in the survival rates of the 3-Step rehydration procedure. This may be due to the sucrose solution to which the embryos were initially exposed also contained the respective cryoprotectant, thus easing the osmotic gradient changes. The osmotic changes encountered with the glycerol by 6-Step rehydration procedure were smaller, thus putting less stress on the embryo, and therefore resulting in higher survival rates when compared with the DMSO by 6-Step rehydration procedure.

The dehydration procedure by Quality Grade (Q.G.) interaction indicates that higher Q.G. embryos survive freeze-thawing more often than lower quality embryos. Quality Grade 2 embryos survive more often ($P<.05$) if they are dehydrated by the T.I.T. procedure versus the 3-Step and embryos dehydrated by the 3-Step process survive more often if they are Q.G. 1 embryos than Q.G. 2 embryos ($P<.05$).

Effects on Quality Grade After Thawing and After Rehydration

The most significant factor affecting the interval from post-dehydration to post-thawing was the initial Q.G. of the embryo. The results showed that the Q.G. 1 embryos had significantly better quality

scores after thawing than did Q.G. 2 or 3 embryos (P=.002). Embryos frozen in DMSO tended to have better quality scores after thawing, thus indicating less cellular damage, than did embryos frozen in glycerol (P=.02). That difference may be explained by the increased osmolarity in the DMSO freezing solutions. The thaw bath temperature had no significant effect during the interval post-dehydration to post-thaw (Table 4).

TABLE 4. QUALITY GRADE EFFECTS FROM POST-DEHYDRATION TO POST-THAWING

Factor	(n)	Least Squares Means of Quality Grade Post-Thawing
Quality Grade Post-Dehydration		
Quality Grade 1	125	2.22[c]
Quality Grade 2	37	2.84[d]
Quality Grade 3	24	3.09[d]
Cryoprotectant		
DMSO	80	2.52[a]
Glycerol	106	2.91[b]
Thaw Temperature		
27 C	88	2.68
37 C	98	2.76

[a, b]Means with different superscripts are different (P=.02).
[c, d]Means with different superscripts are different (P=.002).

Neither the cryoprotectant nor the rehydration procedure had an effect (P>.20) on the change in Q.G. from post-thawing to immediately post-rehydration. Quality Grade 1 embryos post-thawing had a lower Q.G. post-rehydration than Q.G. 2 (P=.011), and Q.G. 3,4 or 5 embryos (P<.001). Quality Grade 2 embryos had lower scores than Q.G. 3,4 or 5 embryos (P<.001). Quality Grade 3 embryos had lower scores than Q.G. 4 or 5 embryos (P<.003) and Q.G. 4 embryos had lower scores than Q.G. 5

embryos (P=.01) (Table 5).

TABLE 5. QUALITY GRADE EFFECTS FROM POST-THAWING TO POST-REHYDRATION

Factor	(n)	Least Squares Means of Quality Grade Post-Rehydration
Cryoprotectant		
Glycerol	104	3.10
DMSO	79	3.13
Rehydration Procedure		
6-Step	81	3.03
3-Step (sucrose)	64	3.08
1-Step (sucrose)	38	3.23
Quality Grade Post-Thawing		
Quality Grade 1	44	1.82[a]
Quality Grade 2	45	2.27[a]
Quality Grade 3	62	3.11[b]
Quality Grade 4	26	3.69[c]
Quality Grade 5	6	4.68[d]

[a, b, c, d]Means with different superscripts are different (P<.01)

The embryos tended to deteriorate as the freeze-thaw process progressed, indicating that every step of the proscess tended to cause more damage to the morphology of the embryo or that degeneration was progressing from some initial damage. However, the actual cooling or freezing process versus the dehydration, crystallization or thawing process may not have caused any damage. Douzou (1977) alluded to the possibility of protein repair during the freezing process. Theoretically the displacement of the equilibrium "Native State <<>> Denatured State" would depend on heat loss or heat gain. During the freezing process (heat loss) previously denatured proteins, i.e. cell membranes, DNA etc.. would be converted to the native state or "repaired"! The challenge therefore is to develop dehydration and warming processes that

would somehow circumvent the inevitable denaturization process.

Conclusions

The most important factor affecting freeze-thaw survival is the initial quality of the embryo. The optimum stage of development was not fully evaluated for a wide range of developmental stages and thus can only be applied in the narrow range from the early blastocyst to the expanded blastocyst, where no significant differences were found. The cryoprotectants, even though they had a difference in osmolarity of 304 moSm, appear to be of equal value in embryo survivability.

The most influential factor affecting the Q.G. through the intervals post-dehydration to post-thawing and post-thawing to post-rehydration was the preceding quality of the embryo. This indicates that the higher the initial quality of the embryo the higher its' quality will be after the freeze-thaw and/or rehydration process.

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APPENDIX

APPENDIX TABLE 1. SYNCHRONIZATION AND SUPEROVULATION REGIME

Day	Treatment
-28	Luteolytic Agent[1]
-17	Luteolytic Agent[1]
-14	Expected Heat
-5	A.M. 5mg FSH P.M. 5mg FSH
-4	A.M. 5mg FSH P.M. 5mg FSH
-3	A.M. 5mg FSH P.M. 5mg FSH
-2	A.M. 5mg FSH + Luteolytic Agent[2] P.M. 5mg FSH + Luteolytic Agent[3]
-1	A.M. 5mg FSH P.M. 5mg FSH
0	Expected Heat and Breeding
+7	Collection
+11	Luteolytic Agent[1]
+15	Expected Heat
+36	Comparable to day -14

[1]500mcg Estrumate or 25mg Lutalyse.
[2]750mcg Estrumate or 35mg Lutalyse.
[3]250mcg Estrumate or 10mg Lutalyse.

APPENDIX TABLE 2. NONSURGICAL EMBRYO COLLECTION FLUSHING MEDIUM

Component	Final Concentration (% V/V)	
Phosphate Buffered Saline[1]	97.09	
Fetal Calf Serum[1]	1.94	
Antibiotics[1]	.97	
Penicillin		97.08 Units/ml
Fungizone		.24 mcg/ml
Streptomycin		97.08 mcg/ml

[1]Gibco Labatories.

APPENDIX TABLE 3. SHORT-TERM CULTURE MEDIUM

Components	Final Concentration (% V/V)	
Phosphate Buffered Saline[1]	77.67	
Fetal Calf Serum[1]	21.55	
Antibotics[1]	.78	
Penicillin		77.66 Units/ml
Fungizone		.19 mcg/ml
Streptomycin		77.66 mcg/ml

[1]Gibico Laboratories.

APPENDIX TABLE 4. THREE-STEP DEHYDRATION SOLUTION

DMSO				
Concentration	0.00M	0.50M	1.00M	1.50M
%(v/v) STCM[1]	100.0	66.7	33.3	0.0
%(v/v) DMSO Freezing Medium[2]	0.0	33.3	66.7	100.0

Glycerol				
Concentration	0.00M	0.47M	0.93M	1.40M
%(v/v) STCM[1]	100.0	66.7	33.3	0.0
%(v/v) Glycerol Freezing Medium[2]	0.0	33.3	66.7	100.0

[1]Appendix Table 3.
[2]Appendix Table 7.

APPENDIX TABLE 5. TIMED INTERVAL TITRATION MEDIUM (STOCK SOLUTION)

Glycerol[1]		DMSO[3]	
Ingredient	(%v/v)	Ingredient	(%v/v)
Short Term Culture Medium[2]	85.0	Short Term Culture Medium[2]	84.0
Glycerol (100%)	15.0	DMSO	16.0

[1]Final concentration 2.10M.
[2]Appendix Table 3.
[3]Final concentration 2.25M.

APPENDIX TABLE 6. TIMED INTERVAL TITRATION, DEHYDRATION PROCEDURES

Glycerol	DMSO
5ml of STCM[1]	5ml of STCM[1]
Add:[3]	Add:[3]
1.40ml of a 2.10M glycerol solution[2] 0.46M glycerol	1.40ml of a 2.25M DMSO solution[2] 0.49M DMSO
Add:[3]	Add:[3]
2.50ml of a 2.10M glycerol solution[2] 0.95M glycerol	2.60ml of a 2.25M DMSO solution[2] 1.00M DMSO
Add:[3]	Add:[3]
6.10ml of a 2.10M glycerol solution[2] 1.40M glycerol	6.00ml of a 2.25M DMSO solution[2] 1.50M DMSO

[1]Appendix Table 3.
[2]Appendix Table 7.
[3]Add in ten minute intervals.

APPENDIX TABLE 7. STOCK FREEZING MEDIUM

	STCM[1](% V/V)	Cryoprotectant(% V/V)
DMSO 1.5M	89.00	11.00
Glycerol 1.4M	90.00	10.00

[1]Appendix Table 3.

APPENDIX TABLE 8. SIX-STEP REHYDRATION PROCEDURE

DMSO							
Concentration	1.50M	1.25M	1.00M	0.75M	0.50M	0.25M	0.00M
%(v/v) STCM[1]	0.0	17.7	33.3	50.0	66.7	82.3	100.0
%(v/v) Freezing Medium[2]	100.0	82.3	66.7	50.0	33.3	17.7	0.0

Glycerol							
Concentration	1.40M	1.17M	0.93M	0.70M	0.47M	0.23M	0.00M
%(v/v) STCM[1]	0.0	17.7	33.3	50.0	66.7	82.3	100.0
%(v/v) Freezing Medium[2]	100.0	82.3	66.7	50.0	33.3	17.7	0.0

[1]Appendix Table 3.
[2]Appendix Table 7.

APPENDIX TABLE 9. SUCROSE REHYDRATION SOLUTIONS 0.5M

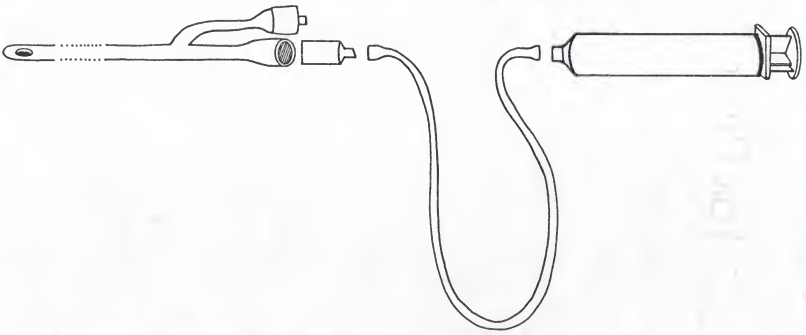
STCM[1] or Cryoprotectant[2] (ml)	Sucrose (grams)
10.0	1.71

[1]Appendix Table 3.
[2]Appendix Table 7.

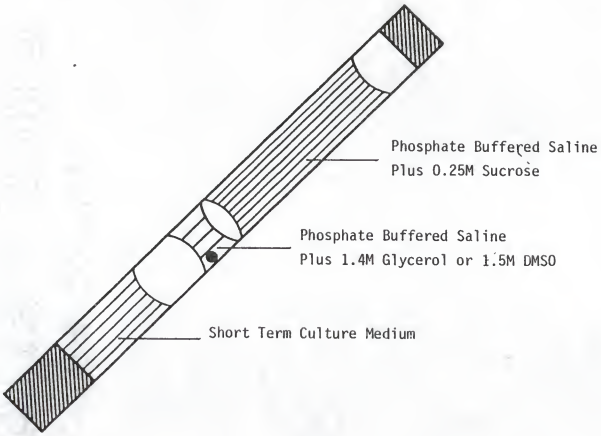
APPENDIX TABLE 10. THREE-STEP REHYDRATION PROCEDURE

Glycerol			
1.4M glycerol[1]	>>>	1.4M glycerol + 0.5M sucrose	>>> 0.5M sucrose >>> STCM[2]
DMSO			
1.5M DMSO[1]	>>>	1.5M DMSO + 0.5M sucrose	>>> 0.5M sucrose >>> STCM[2]

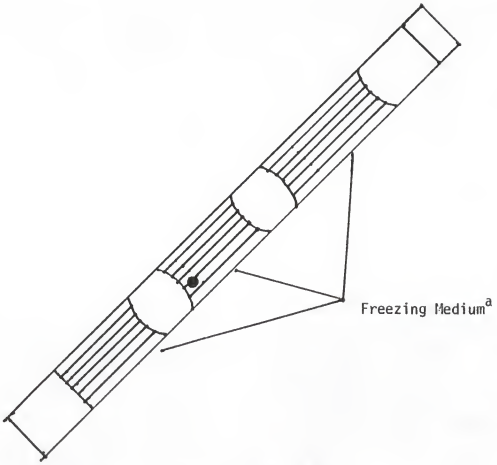
[1]Appendix Table 7.
[2]Appendix Table 3.



APPENDIX FIGURE 1. TWO-WAY FOLEY FLUSHING APPARATUS

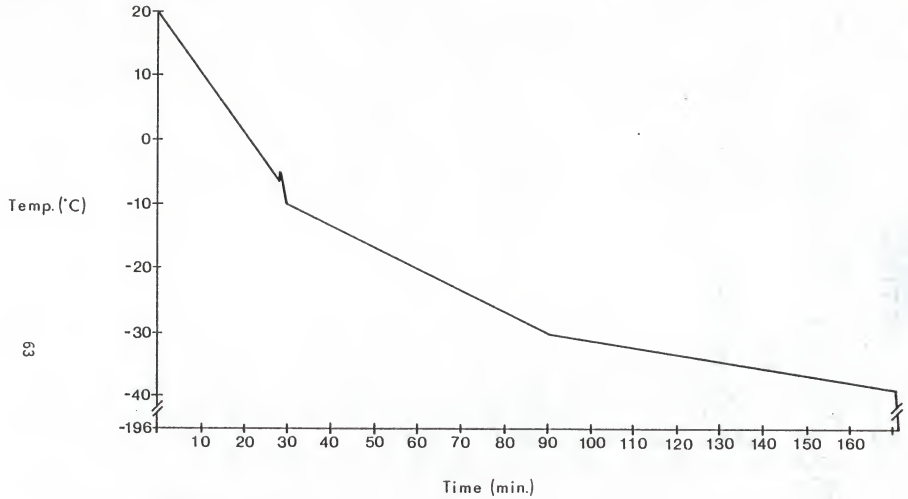


Appendix Figure 2. 1/2ml 1-Step Straw

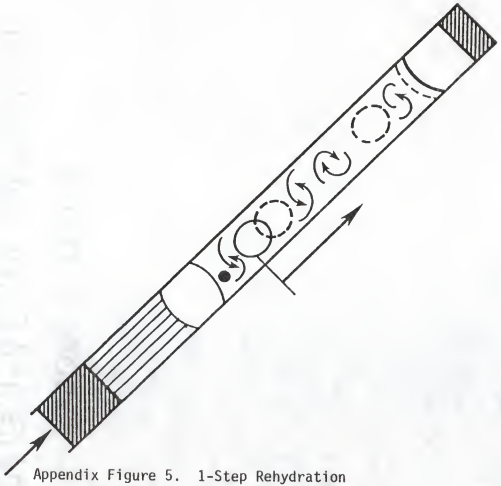


Appendix Figure 3. 1/4ml Straw

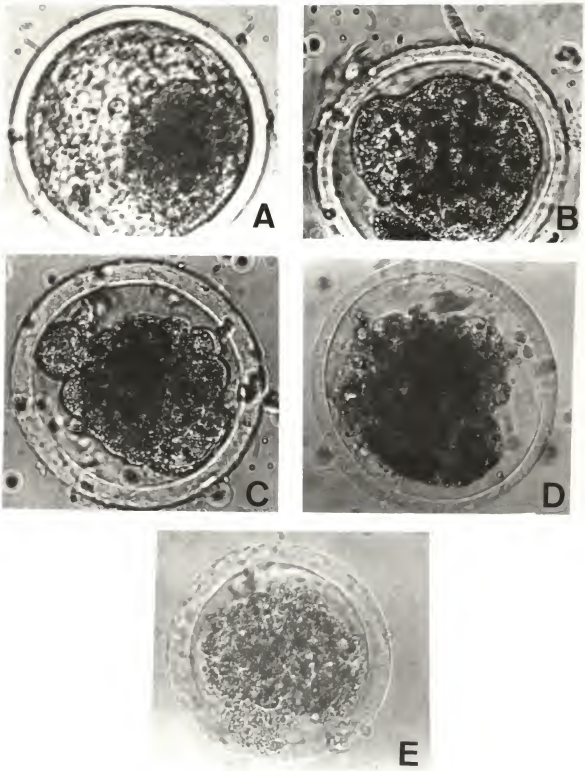
[a]Appendix Table 7



Appendix Figure 4.
Cooling Rate For Freezing Bovine Embryos



Appendix Figure 5. 1-Step Rehydration



APPENDIX FIGURE 6. EMBRYO QUALITY CLASSIFICATIONS.
A. Grade 1 expanded blastocyst. B. Grade 2 blastocyst.
C. Grade 3 blastocyst. D. Grade 4 collapsed blastocyst.
E. Grade 5 degenerate.

DEVELOPMENT AND EVALUATION OF CRYOPRESERVATION TECHNIQUES FOR BOVINE EMBRYOS

by

RANDALL SCOTT PRATHER

B.S., Kansas State University, 1982

AN ABSTRACT OF A MASTER'S THESIS

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ABSTRACT

Several procedures for cryopreservation and the factors affecting cryopreservation of embryos are reviewed. A total of 228 embryos were non-surgically collected from superovulated cows. The embryos were then dehydrated in DMSO or glycerol by a 3-Step procedure or a timed interval titration procedure. They were frozen to -196 C and thawed in a 27 C or 37 C water bath and rehydrated by a 6-Step, 3-Step (sucrose) or 1-Step (sucrose) procedure. This yielded a 2 X 2 X 2 X 3 factorial treatment structure. Embryos were graded according to stage of development post-dehydration, then were quality graded post-dehydration, post-thawing, and 10 min post-rehydration. Survival was based on development after 12 h in vitro. Neither the cryoprotectant, dehydration procedure, thaw temperature, rehydration procedure, number of days frozen, breed, sire or dam of the embryo nor the stage of development (early blastocyst, blastocyst, expanded blastocyst) had any significant effect on survival ($P>.10$). The only significant single factor was the initial quality grade of the embryo. Quality grade 1 and 2 embryos survived more often than quality grade 3 embryos ($P<.05$). Interactions indicated that the 3-Step renydration procedure resulted in higher survival rates if the thaw temperature was 27 C ($P<.05$). Embryos more advanced in development and better quality survived more often ($P<.05$) than less developed and lower quality embryos. Freezing in glycerol resulted in survival more often if the embryos were rehydrated by the 6-Step procedure versus the 1-Step procedure ($P<.05$). For the interval post-dehydration to post-thawing using DMSO as the cryoprotectant resulted in better quality scores ($P=.02$). For both intervals post-dehydration to

post-thawing and post-thawing to post-rehydration the previous quality grade was significant in determining the new quality grade ($P<.01$). At each step of the freeze-thaw process the embryos became progressively less morphologically intact.