BASIC ASPECTS OF CRYOPRESERVATION OF BOVINE EMBRYOS

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Introduction

The cryopreservation of bovine embryos is an established procedure. Since the first report of the successful freezing of a bovine embryo by Wilmut and Rowson in 1973, numerous reports have been published describing improvements and modifications of the methods used to freeze and thaw bovine embryos. Undoubtedly, the actual figure may be much higher. The methods used to cryopreserve embryos have been well described, and are even taught in short workshops. Recent comparisons of details of these methods have been published. In fact, given the pressures of the commercial marketplace to produce "results", there is often little time or opportunity to test innovations, especially on a large scale. Perhaps an examination of the "basics" of embryo cryopreservation might prompt a cautious trial of some of the newer methods that have been described for the embryos of laboratory species. That is the purpose of this brief review. Furthermore, as innovative methods to produce bovine embryos become more wide-spread, methods such as in vitro maturation of ovarian oocytes and in vitro fertilization(IVF) of mature oocytes, older procedures of cryopreservation may prove inadequate. Only an appreciation of fundamental principles is likely to lead to success.

The cryopreservation of embryos can be considered as the application of these fundamental principles together with allowance for certain practical considerations. Although this review is intended to address bovine embryos specifically, certain lessons can be learned from the results with oocytes embryos of other species as well. Where appropriate, therefore, such results will also be cited. At present, embryos are cryopreserved either by slow, equilibrium cooling methods, or by rapid, non-equilibrium cooling. Regardless of whether embryos are cryopreserved by slow or rapid cooling, certain fundamental aspects of embryo cryopreservation involve the same factors. These include:

1. Embryo stage and grade.
2. Type and concentration of cryoprotective additive(CPA)
3. Cooling rate
4. Storage in liquid nitrogen(LN_2)
5. Warming rate.
6. Dilution or removal of CPA

Practical considerations encompass factors common to all methods of embryo cryopreservation as well as those unique to ET. These include:

1. Sterility of CPA solutions.
2. Embryo container.
3. Time required for freezing.
4. Freezing "machine" or instrument.
5. Identification of embryos
6. Handling of thawed embryos prior to transfer.

Fundamental Principles

1. The Embryo : The first variable to be considered in bovine embryo cryopreservation is the embryo itself. In general, the same methods are used to cryopreserve embryos from both beef and dairy cattle. Although pregnancies have been achieved by the transfer of frozen-thawed embryos of both types of cattle as well as for mixed-breed embryos, there really has not been a comprehensive analysis of this question. Unfortunately it is difficult to unravel the many variables that may influence ultimate pregnancy by embryo transfer in cattle, aside from the question of cryopreservation. These variables include(1) the quality of the embryos being transferred, (2) the fecundity of both the donor cow and of the recipient, (3) the skill of the ET "technician", (4) the hormone regimen used to induce superovulation of the donor, (5) estrous synchrony between the donor and recipient cows, and (6) the selection of either hormonally-induced or natural estrous recipients. It may be that there are not differences in the sensitivity to cryopreservation of embryos from different types of cattle. However, it has been reported that embryos from different strains of laboratory mice do exhibit such differences it is possible that of various cattle breeds could result in differential sensitivity. It is known, for example, that the spermatozoa of different human males exhibit differences in their freezing sensitivity. Therefore, there may be significant differences between embryos of different breeds.

Nevertheless, presumptive evidence suggests that embryos from different donor cows do not differ in their freezing sensitivity. For example, the results in Table 1 show the pregnancies from donors whose embryos were collected and frozen on multiple occasions. Although pregnancy rates for individual collections ranged from lows of to 20% to highs of 64 to 80%, the data suggest that when the sample size for different donors is large enough for meaningful comparison, all of the donors yielded pregnancy rates of about 45%.

It has often been suggested that there are differences in the freeing sensitivity of embryos of different grades or "quality". A practical consequence of this view is that only embryos of the best grades are actually frozen. Poor quality embryos may even be discarded rather than being frozen to yield unacceptably low pregnancy results. For fresh embryos, it is well established that the likelihood of pregnancy is

Table 1. Pregnancies by Transfer of Frozen-Thawed Bovine Embryos

Donor	Collection	#Preg/#Thaw by Collection	#Preg/#Thaw by Donor	Pregnancy(%)
A	1	7/12	8/19	42
	2	1/5		
	3	0/2		
B	1	7/20	12/32	38
	2	2/3		
	3	4/10		
C	1	0/2	8/17	47
	2	4/5		
	3	3/9		
D	1	7/11	11/26	42
	2	3/10		
	3	1/5		
E	1	2/3	6/11	55
	2	2/4		
	3	2/4		
F	1	4/14	14/40	35
	2	3/8		
	3	5/8		
	4	2/10		
Total			59/145	40.7

highly correlated with the morphological quality of the embryos. similar observations have been reported even for human embryos. but a comparison of pregnancy results between fresh and cryopreserved embryos based on their grade prior to freezing suggests that this conclusion may not be justified. That is, the percentage pregnancy of frozen embryos is indeed less that of fresh embryos; but the proportionality of pregnancy to embryo grade appears to be the same for both fresh and frozen embryos. The embryonic stage has also been considered as an important variable of cryopreservation. In practice at present, most commercial ET firms collect bovine embryos non-surgically between days 6 to 8 after artificial insemination, so that the stages frozen range from early morulae(~32 to 64 blastomeres) to expanded blastocysts (> 100 to 175 blastomeres with enlarged blastocoel cavities). The results in Table 2 for pregnancies produced by the direct transfer of bovine embryos of different stages (and of excellent to poor quality before freezing) cryopreserved in glycerol suggest that embryos of different stages may not differ significantly in their freezing sensitivity.

Table 2. Pregnancies from Frozen-thawed Bovine Embryos with Respect to Embryo Stage when Frozen. All Grades of a Given Stage were Pooled.

Embryo Stage	Pregnant/Thawed	Pregnant(%)
Late Morula	168 / 398	42.2
Early Blastocyst	103 / 272	37.9
Late Blastocyst	43 / 112	36.8

As embryos of early developmental stages become available through IVF, it is possible that significant differences in freezing sensitivity between stages will become apparent. These may be due to differences in the permeability characteristics of various embryonic stages of development.

2. Cryoprotective additives (CPAs) : Several different solutes have been used to protect bovine embryos against freezing damage, including dimethyl sulfoxide(DMSO), glycerol, and propylene glycol. These cryoprotectants share many common features. They have low molecular weight and high solubility in water, are not toxic to embryos, and permeate embryos rather quickly. Their low molecular weights and high solubility mean that they depress the freezing points of solutions to temperatures as low as -40℃ to -50℃ or below. Thus, during slow cooling there is liquid present in the solution to those temperatures, allowing time for the embryo to dehydrate during cooling. Their low toxicity means that embryos are not damaged simply by exposure to molar concentrations of these solutes. It must be realized, however, that extended exposure of embryos to high concentrations of these solutes at 20℃ or above means that the embryos will begin to metabolize in the presence of non-physiological concentrations of the cryoprotectant. This is not evidence of solute toxicity per se, but rather only that embryos may be damaged by undergoing development in such CPAs. However, embryos that have become permeated by these cryoprotectants may become sensitive to osmotic shock.

Until about 5 years ago, most bovine embryos were frozen in a solution of about 1.5 Molar glycerol. Comparisons of the effects of step-wise vs single-step addition of CPAs on the survival of cryopreserved bovine embryos have found little or no difference between then. However, when embryos are rapidly diluted directly out of a concentrate solution of some cryoprotectants, such as glycerol, after freezing the may be damaged by osmotic shock. That is the phenomenon exhibited by all mammalian cells in which the intracellular solution is more concentrated than the extracellular solution. Water enters the cell more rapidly than an intracellular solute, such as glycerol, can leave. Depending on the relative permeabilities of the embryo to water and to the solute, cell volume may increase to volume that the embryo can not tolerate, and the embryo bursts. Bovine embryos are relatively impermeable to glycerol, which renders them sensitive to osmotic shock when they are directly diluted

out of a glycerol solution into isotonic saline. They are even more sensitive to osmotic shock when diluted out of dimethyl sulfoxide (DMSO) than out of glycerol, however. That difference in permeability of bovine embryos to glycerol vs DMSO illustrates one important aspect of cryoprotectants. They may exert equivalent protection during freezing. But if the embryo is much more permeable to one CPA than to another, then it will be much less sensitive to osmotic shock when diluted out of the former compared to the latter. Since 1992, more and more bovine embryos have been cryopreserved in the presence of either ethylene glycol or propylene glycol. Both of these glycols have rather low molecular weights, are very soluble in PBS, depress the freezing point of solutions to -40℃ or below, and are not toxic to embryos. This means that embryos can be efficiently cryopreserved in 1 to 2 Molar solutions of glycols. More importantly, mammalian embryos are extremely permeable to glycols. This means that after such cryopreserved embryos are thawed, they can be transferred directly into recipient cows without the risk of osmotic shock. Use of ethylene glycol to cryopreserve bovine embryos has become extremely common during the past few years. In practice, it means that bovine embryos can be transferred in a fashion very much like artificial insemination.

3. Seeding of Samples : The theory and practice of seeding in embryo cryopreservation has recently been described in some detail. Suffice it to say that this is an important, yet often misunderstood, step in embryo cryopreservation. Briefly, it is a method by which a solution that has been cooled below its freezing point is induced to undergo crystallization under controlled conditions. When an aqueous solution is cooled below 0℃, it will spontaneously freeze. However, the temperature at which this spontaneous crystallization occurs may vary widely from as high as -30℃ to as low as -20℃. Seeding produces a phase change of the aqueous solution that causes an increase in solute concentration. Embryos respond osmotically by the loss of cell water. Small differences between seeding temperatures, e.g. from -4℃ to -10℃, have little effect on embryo survival if sufficient time after seeding is allowed for embryos to undergo osmotic contraction. Furthermore, there is mo evidence to support the contention that the release of the latent heat of fusion resulting from crystallization at seeding damages embryos. Seeding of embryo samples may be induced manually, automatically by the freezing instrument itself, or even by the inclusion of crystals or other chemicals within the straw in which embryos are being frozen. It should also be recognized that spontaneous crystallization also depends on the sample volume. Therefore, embryos frozen in volumes of <0.1 ml or less in straws will tend to supercool, deliberate seeding must be used. Embryos frozen in volumes of >0.5 ml or greater will occur spontaneously at rather high subzero temperatures. Whatever the method, seeding seems to be required for high survival of embryos that are cooled at low rates.

4. Cooling rates to Intermediate Subzero Temperatures : The first reliable method to cryopreserve bovine embryos by Willadsen in 1987 specified the use of cooling from -6℃ to -35℃ at a rate of 0.3℃/minute. Since then, the majority of reports have followed that same practice. Only a few authors have examined the

effects of higher cooling rates on the functional survival of bovine embryos. Recent studies have confirmed that maximum survival of bovine embryos seems to be achieved with very low cooling rates. One important practical consequence of the use of a cooling rate of 2℃ vs 0.3℃/minute from -7℃ to -35℃ is that the latter would require about 95 minutes whereas the former would only require 14 minutes.

Despite that apparent advantage, very low cooling rates continue to be used to cryopreserve bovine embryos.

5. "Plunge Temperatures" : Considerable attention has been directed to examination of the effects of the temperature at which slowly cooled embryos are plunged into liquid nitrogen Again, Willadsen's original procedure of cooling bovine embryos below -35℃ at a rate of 0.1℃/minute seems to have been followed slavishly. In fact, he has stated that his intention was simply to maintain a constant temperature of -35℃ for thirty minutes, so as to permit the embryos to equilibrate osmotically. The freezing instrument that he used at that time did not permit a cooling rate of 0℃ /minutes, ie no cooling at all. A rate of 0.1℃/minute was the lowest rate available. Nevertheless, many experiments have since been conducted to compare cooling at that low rate from about -35℃ to lower temperatures. In general, the results of these experiments have been inconclusive. This is not surprising in light of the fact that maximum concentration of the extracellular solution, and therefore, maximum osmotic contraction of the embryo will have occurred at much higher subzero temperatures. In short, attention to fundamental principles leads to the conclusion that slow cooling may be terminated over a broad subzero temperature range of about -25℃ to -40℃ with the same overall results. Experimental data have confirmed those predictions. Again, the practical implication is the shortening of the time required for the successful cryopreservation of bovine embryos.

6. Storage of Cryopreserved Embryos : although frozen embryos will retain high viability if stored at -80℃ for up to about 5 days., long-term storage requires that the embryos be stored below about -130℃, the glass transition temperature of water.

In practice, the easiest and safest method is to store cryopreserved embryos in liquid nitrogen at -196℃. Mouse embryos stored at that temperature exhibit the same high survival after 24 hours or after more than 15 years. In fact, live young mice have been produced from cryopreserved embryos stored for more than 13 years in liquid nitrogen. It is important to appreciate that cryopreserved embryos, especially those frozen in plastic straws, are exceedingly sensitive to even very brief exposure to room temperature. For example, a 1/4 ml plastic straw will warm from -196℃ to above -100℃ in less that 5 seconds. If a frozen embryo in warmed briefly and then plunged back into liquid nitrogen, it will undoubtedly be irreversibly damaged. Thus, the important thing about embryo storage is to be absolutely certain that cryopreserved embryos are always kept submerged in liquid nitrogen until the very moment at which they are to be warmed and thawed.

7. Warming Rates : Just as there are optimum cooling rates for maximum survival, so are there optimum warming rates. In general, embryos cooled slowly to high

subzero temperatures of about -30℃ to -40℃ before being rapidly cooled to -196℃ require rather rapid warming of about at least 200 to 350℃/minute for maximum survival. Embryos cooled slowly to -60℃ or below require rather slow warming of about 25℃/minute or less. It is important to recognize the influence of the sample volume, the embryo container, and the cryoprotectant itself on the response of bovine embryos to warming rates. An embryo frozen in a volume of about 0.5ml in a glass ampoule will warm at about 350℃/minute when placed directly into a warm water bath However, an embryo frozen in a volume of about 0.05 ml in a plastic straw will war, at about 2,500℃/minute when similarly treated. There are important biological consequences of these different warming rates. Furthermore, it should also be appreciated that a frozen embryo held in air for about 10 or 15 seconds before being placed into a warm water bath experiences a substantially different set of warming rates than one that one that is directly and immediately plunged into warm water from liquid nitrogen. If frozen in glycerol, embryos warmed at 200℃/min will survive, whereas those frozen in ethylene glycol will not. In contrast, embryos frozen in ethylene glycol will exhibit higher survival when warmed rapidly than will those frozen in glycerol.

8. Dilution of Cryoprotectants : This is the last but often over-looked step of embryo cryopreservation. Embryos are cryopreserved in CPAs ranging in concentration from 1 to 8 Molar. Virtually all CPAs permeate embryos prior to their being cooled. In general, if embryos are rapidly diluted out of CPAs, they will be subjected to osmotic shock, resulting in embryo damage or death. A common practice has been to dilute cryopreserved embryos out of the in a slow step-wise fashion. Although this method works in practice, it is usually rather slow and laborious. A more efficient and shorter method is to use a non-permeating solute such as sucrose as an osmotic buffer to lessen the chance of an osmotic shock It has been demonstrated that this single-step dilution can be performed within the straw in which the embryo was originally frozen. A variation on this theme has been to mix sucrose with the CPA to lessen the osmotic shock, and to perform a two-or three-step dilution of the CPA. Undoubtedly, more efficient methods to recover cryopreserved embryos from CPAs will be introduced in the future. In short, application of certain fundamental principles may well improve the efficiency and survival of cryopreserved bovine embryos.

Practical Considerations

1. Sterility : Common sense dictates that aseptic technique be used in all aspects of embryo transfer. Certainly, all media including CPAs in which embryos are to be suspended should be sterile. But it should also be appreciated that most CPAs are used in such high concentrations that it is unlikely that microorganisms will grow rapidly. Furthermore, embryos will normally be exposed to CPAs for rather short times before being cooled to below 0℃. This means that there will actually be only

a very short time during which contaminants present in CPAs might actually grow and divide in such solutions. Far more riskier are the isotonic solutions containing sera of other proteins that are commonly used to rinse or collect embryos. Great care should be exercised to maintain the sterility of those solutions. Even when held at 4℃ in a refrigerator, such solutions can easily become contaminated and can support the growth of a wide variety of microbial forms. Once contamination is suspected, e.g. because of suspicious turbidity, the entire batch of solution should be discarded.

2. Embryo Containers : Initially, embryo were commonly cryopreserved in small glass ampoule. Later, plastic freezing vials were substituted. About 15 years ago, the plastic straws used to freeze bovine semen were introduced for the cryopreservation of embryos as well. Each type of container has certain advantages and disadvantages. Glass ampoules are easily sterilized and have good thermal conductivity, but, they are difficult to seal completely, and may explode during thawing. Plastic vials are easy to seal securely, but have poor thermal conductivity, so that they can not be warmed rapidly even when submerged in warm water baths. Plastic straws are easy to seal and occupy less storage space. But their thin walls mean that they cool and warm very rapidly. Embryos in straws can be easily damaged by excessive cooling during seeding, or can be damaged by inadvertent warming during handing after the sample is frozen. Most importantly, allowance must be made for the type of container being used for a given procedure. It was noted above that the warming rates that are achievable when a frozen sample is placed directly from liquid nitrogen into warm water vary considerably. Such differences may produce very different survivals of embryos.

3. Time for Freezing : As noted above, small differences in the cooling rates may yield large differences in the time required for embryo cryopreservation. In a commercial context, "time is money". Yet again, however, common sense dictates that small time savings may be very expensive if the overall pregnancy results are poor. Therefore, caution suggests that shortening of the time used for freezing should not be exercised if there is the possibility of jeopardizing embryo integrity. This may be especially true of newer methods of non-equilibrium embryo cryopreservation. Rapid cooling methods may only require minutes to perform. But these methods are less "tolerant" of small mistakes than are the more conventional slow cooling methods. Furthermore, continuous slow cooling at 2℃ /minute only requires about 15 minutes to perform, a time not too much longer than that required for the processing of embryos for rapid cooling.

4. Freezing Instruments : A wide variety of reliable and excellent instruments have been manufactured for the cryopreservation of mammalian embryos. In some, the sample compartment is cooled with tempered gas, in others, the chamber is filled with alcohol that is in turn cooled b mechanical refrigeration, in still others, the chamber is cooled by balancing a heater in the chamber against cooling provided by liquid nitrogen; and in some, the chamber is cooled by a thermoelectric device.

All have been used successfully for embryo cryopreservation. All have both advantages and disadvantages. All else being equal, the least expensive and most reliable instrument that is least susceptible to mechanical or electrical failure would seem to be the instrument of choice. Considerations such as availability of electrical power, weight and mobility, electrical and mechanical reliability, and the numbers of samples that can be frozen at any one time should also be borne in mind when a freezing instrument is purchased. More important than the freezer itself is that understanding of fundamental principles of cryobiology be exercised when the instrument is used to cryopreserve embryos.

5. Other Practical Considerations : Success in embryo cryopreservation is usually measured in terms of the numbers of pregnancies produced by the transfer of frozen embryos. But other factors must also be considered. For example, great care must be exercised to assure correct identification of each embryo. The owner of a herd of valuable pure-bred cattle is unlikely to be impressed by a high pregnancy rate from frozen embryos if his recipients give birth to a motley collection of cross-bred calves nine months after embryo transfer. Careful record-keeping and accurate statistical analyses of results are equally important. The efficiency of embryo cryopreservation should always be measured against the potential calf crop of a given donor cow if the embryos had not been cryopreserved. In general, the principal goal of embryo transfer is to enhance the genetic output of specific donor-sire matings. Therefore, it seems most important that the ultimate tabulation of embryo cryopreservation should be calculated in terms of the number of live calves produced from frozen embryos of a given collection compared to what might have been achieved if the embryos had not been frozen.

Conclusions

Great progress has been made in the cryopreservation of bovine embryos. Entire herds have been produced by the transfer of frozen-thawed embryos. The methods used to freeze and thaw embryos have become simpler and more reliable. Most importantly, substantial progress has been made in understanding the entire phenomenon of embryo cryopreservation. As new and dramatic improvements in related aspects of bovine reproduction are introduced into field application, embryo cryopreservation will become increasingly important. For example, large-scale production of genetically-engineered cattle from cloned and transgenic embryos that were the result of in vitro matured and fertilized ovarian oocytes will only be commercially feasible when such valuable embryos can be cryopreserved with little or no loss of their functional viability. Appreciation of both the basic and practical aspects of embryo cryopreservation will contribute to success in these new applications of cattle husbandry.