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Cryopreservation of Bovine Embryos : State of the Art

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Introduction

The first scientific research in artificial insemination of domestic animals was conducted in 1780 by an Italian physiologist Spallanzani. He was the first to demonstrate the possibility of artificial insemination employing extended semen. He also found that freezing stallion semen did not kill the sperm cells but held them in a dormant state until exposed to heat. Since Spallanzani's early work, researchers have been constantly working to develop new techniques and to formulate new extenders for the handling of spermatozoa. Mammlian spermatozoa were frozen succesfully to dry-ice temperatures in 1950 by Polge et al., and this is considered the beginning of the modern cryobiology. The low temperature preservation of mammalian sperm led quickly to the successful freezing of other mammalian cells, and it greatly influenced the cattle industry. This also prompted research interest in freezing mammalian ova and embryos, however, nearly a quarter of a century passed before proper techniques were developed.

Success was first reported in 1972 by Whittingham et al. for mouse embryos and shortly after by Wilmut (33) in an independent study, and four years later for cattle embryos by Willadsen (35). The time leg between Polge's and Willadsen's findings provides once again an example of the need of interplay between basic and applied research. Today, much of the research has been conducted with embryos from laboratory species and the results have been applied to embryos from domestic animals. Some research has also been conducted with bovine embryos and high pregnancy rates with frozen-thawed bovine embryos have been obtained.

To achieve high pregnancy rates with frozen thawed bovine embryos several steps need to be done properly. This paper reviews and discusses each of these steps: flushing media, embryo stage and quality, "washing" of the embryo, method of glycerolization and deglycerolization, and cooling, freezing, and thawing procedures.

Flushing and Incubation Media

A variety of media, ranging from complex tissue culture media (TCM-199 and MEM) and chemically defined media BMOC-3, Hams's F-10, or Menezo's) to simple balanced salt solutions (phosphate buffered saline solution PBS or Ringer's) with serum or bovine serum albumin, have been used for the collection and incubation of embryos. However, PBS with minor modifications is the solution most commonly used for flushing, freezing and thawing bovine embryos.

Inorganic salts: There is no research published that indicates the metabolic requirements of bovine embryos for the inorganic salts that are present in PBS. Its main constituent is NaCl (8 gr/lit) which function is to regulate the osmotic pressure of the solution. Mouse embryos develop under a wide range of K^+ concentrations (0.6 mM to 48 mM) but is almost stopped in the absence of K^+ (29). Studies on the uterus and oviductal secretions from domestic animals have indicated high K^+ levels (6, 7, 28, 19). Mouse embryos also develop to blestocysts under a wide range of Ca^{++} ions (0.4 mM to 10 mM), and their development is arrested in its absence (30, 5). It has also been reported that the lack of PO_4, Mg^{++} and SO_4 in the incubation media had little effect on the embryo development to the blastocyst stage (29).

Organic constituents: D-glucose, Na pyruvate and lactate are commonly added to the incubation media. Pyruvate and lactate are the preferred energy sources for early preimplantation embryos, while glucose is incorporated into the embryo at all stages of development in much greater amounts than either pyruvate or lactate (36). Research in the mouse has also suggested that in vitro processes such as hatching, attachment and trophoblast outgrowth are glucose-dependent events.

Colloidal materials such as heat inactivated fetal calf serum, newborn serum or steer serum are also added in amounts that range from 1 to 2 % in the flushing media to 10 to 20 % in the incubation media (1,3). At the present time, there is not evidence indicating which are the beneficial substances in the serum that promote embryonic development. Bovine Serum Albumin (BSA), fraction V, may also be used. BSA has been shown to be necessary for the blastocyst formation in the rabbit (10), however, its role in the development of the bovine embryo has not been explained.

Coligative Properties: The effect of osmotic pressure and pH on bovine embryo development has not been studied in detail. It is known that hyper or hypo tonicity of the extenders affect drastically the fertilizing ability of bovine spermatozoa. The osmotic pressure of solutions used for flushing or incubation of embryos ranges between 270 mOsm/kg to 310 mOsm/kg. Brinster (2) and Naglee et al. (20) reported that 2-cell mouse embryos developed into blestocysts in media ranging from 200 to 354 mOsm/kg, and rabbbit embryos developed through the same stages in solutions ranging from 230 to 339 mOsm/kg, respectively. However, the use of Ringer's solution (osmotic pressure of 230-250 mOsm/kg) as flushing media for collection of bovine embryos affected drastically pregnancy rates (Fahning, unpublished).

The effect of pH has not been studied either. The media is prepared with pH's that range from 7.0 to 7.4.

Embryo Stage and Quality

Earlier reports indicated the difficulty in freezing bovine and mouse embryos at different developmental stages. Bovine embryos in the early stages of development do not survive freezing and thawing very well, however, morulas and blastocysts survived the best. Expanded glastocysts show lower survival rate, nevertheless they may also be frozen and good pregnancy rates may be obtained (8). Post-thaw survival and pregnancy rates are highly and positively correlated with the quality of the embryos before and after freezing.

Washing of the Embryo

Proper washing has been found to be effective in removing high levels of most pathogens from embryos. Lately, a method for doing it has been recommanded (27). This method involves the transfer of embryos in groups of ten or less through len different solutions of PBS and serum making each time a 1:100 fold dilution. This washing should be done prior to freezing when the zona pellucida is still intact. The zona pellucide acts as an effective pathogen barrier and so the application of this technique is very important for the international movement of embryos. It is also recommended that the embryos be examined for cracks in the zon pellucida after washing and before cryopreservation at a not less than 50X magnification. The embryos should be gently rolled in the dish so that all the surfaces of the zona can be observed.

Glycerolization and Deglycerolization of the Embryos

Many compounds have been demonstrated to protect cells against freezing damage. The compounds may be of two types: permeating and no permeating. The former includes glycerol, dimethyl sulfoxide (DMSO), ethylene glycol and 1,2, propanediol, all of them are small molecules that penetrate cell membranes easily. The latter includes large sugar molecules like sucrose, raffinose or polyvinil pirrolidone (PVP). DMSO was used earlier as the cryoprotectant for embryos, however later it was shown that DMSO in combination with glycerol and now glycerol alone at concentrations ranging from 1.0 to 1.5 M provides better protection to

cells during freezing and thawing.

Kasai et al. (11) reported that the sensitivity of embryos to the rates of freezing and thawing is largerly affected by the cryoprotectant used. The rate and temperature at which the cryoprotectant is added to the medium and also the length of equilibration are not critical for the survival of bovine embryos (14, 26, 4). Bovine blastocysts can be equilibrated in 1.4M glycerol (10 % v/v) in only one step of 10 to 30 min. However, slow removal of the cryoprotectant from the embryo after thawing is important. Direct transfers of non-frozen embryos in glycerolated PBS (14) as well as of frozen embryos without glycerol removal have given poor results (34). In most cases, the concentration of cryoprotectant in the medium is increased at room temperature in 0.5 M steps every 10 minutes until the final concentration is attained. For the removal of cryoprotectant after thawing, its concentration is gradually removed in 0.25 M steps by transferring the embryos through PBS solutions of decreasing glycerol concentration until they are back in pure PBS.

In 1982, Renard et al. and very shortly after, Leibo (15), introduced a new method for the removal of glycerol by inclusion of a 0.25 M sucrose solution in the straw. They postulated that the glycerol would diffuse passively to the exterior of the cells and sucrose, which could not permeate the cells, would avoid the osmotic shock during the dilution step. However, a toxic effect of sucrose was shown in higher concentrations. Today, several studies comparing both methods of deglycerolization have been conducted and it has been reported that there is no difference in pregnancy rates (9, 14). Leibo (15, 16) reported 36.7 % pregnancies with bovine embryos frozen in 1/4 cc straws and thawed in sucrose solution. Renard et al. (24) obtained 46.8 % pregnancies after transfer of frozen embryos deglycerolated in sucrose and 57.7 after transfer of non frozen embryos.

If the choice is to deglycerolate the embryos with sucrose, the embryos are transferred after thawing to a solution of 6.6 % glycerol plus 0.3 M sucrose for 5 minutes, then to a 3.3 % glycerol plus 0.3 M sucrose solution for 5 minutes, and finally into 0.3 M sucrose solution for 10 minutes. Later they are transferred into PBS and serum (9). At Cryovatech we have geen using an alternate method of deglycerolization. This method involves the addition of decreasing molar concentrations of glycerol solutions in a stepwise fashion, each time doubling the previous volume.

Tables 1 and 2 show osmotic pressure readings of the different solutions used for the deglycerolization of the embryos and the osmotic pressure of the solution in which the embryos are during the process, respectively. After addition of 0.25 M solution, the embryos are transferred to 0.25 M alone and then to PBS with 15 % heat inactivated fetal calf serum

Table 1. Osmotic Pressure of Modified Dulbecco's Phosphate Buffered Saline (PBS) Glycerol Solutions

Glycerol concentration	Osmotic pressure[1] mOsm/kg.
140 M	2088
1.25 M	1797
1.00 M	1450
0.75 M	1107
0.50 M	832
0.25 M	576

Table 2. Osmotic Pressure of PBS-glycerol Solutions during the Deglycerolation Process

Deglycerolization steps	solution added PBS-glycerol	Volume added (mis.)	Final osmotic pressure[3] mOsm/kg
1[1]	1.40 M	0.25	2088
2	1.25 M	0.50	2665
3	1.00 M	1.00	1635
4	0.75 M	2.00	1379
5	0.25 M	4.00	1121
6	0.25 M	8.00	852
7	Transferred to 0.25 ml		576
8	Transferred to PBS + HIFCS[2]		300

1 Embryos placed in 0.25 ml of a 1.4 M glycerol solution in PBS.

2 HIFCS Heat inectivated fetal calf serum.

3 Direct readings in a osmometer Advanced Instruments model 3WII.

The addition of each solution is done at 5 minute intervals and periodically the petridish is gently aggitated. Following the process described we have obtained 30/51 or 58.8 % pregnancies.

Machanism of action of cryoprotectants: The mechanism of action of cryoprotectants is not fully understood yet. It was initially believed that they acted by changing size and shape of ice crystals formed, changes that presumably reduced their mechanical destructiveness (22).

We know today that the addition of a non-electrolyte like glycerol modifies the solution markedly as a consequence of colligative properties. It reduces the freezing point of a solution. Thus, freezing and specially intracellular ice occurs at lower temperatures in the presence of cryoprotectants. This may give the cells more time to dehydrate during the cooling process. The cryoprotectants may also lessen the high intracellular concentration of molecules that are eeached as cells dehydrate. So, the molecules may produce less damaging effect when they are disolved in the cryoprotectant than if they were alsone. This is called the "salt buffering effect". A third theory is that the cryoprotectants may act at membrane levels during the change of fluid to relatively solid structures and then back to relatively fluid structures again (17, 18).

Cooling, Freezing, and Thawing Procedures

The initial attempts to freeze bovine embryos had very limited success, however in the past few years a successful and reproducible technique has been developed. The major discovery in the freezing technique was the extreme sensitivity of the embryos to rates of freezing and thawing.

Physical events during freezing: Water is the primary consituent of biological fluids. Pure water freezes at 0°C, whereas water containing ions and other substances freezes at lower temperatures. As water in a solution freezes, pure ice cristals form leaving behind greater liquid concentrations of those substances in solution (23). The freezing point of any solution decreases as the concentration of molecules dissolved in it increases. The first ice crystals to form are relatively pure water which leeves the unfrozen water between these crystals with a higher concentration of dissolved substances than originally. As additional water feeezes the remaining solution becomes even more concentrated.

Supercooling and seeding: The freezing point of a solution with cryoprotectant may range between -3°C to -10°C. However, when we cool a solution at slow rates the solutes in the intracellular mass beocme so concentrated that its freezing point decreases several degrees. This is called supercooling. At this point, water can flow out of the cell and freeze externally, thereby concentrating the intracellular solutes. Or, intracellular water can also freeze *in situ* concentrating the intracellular solutes. Which of this two routes will prevail will depend on cooling rate and will be critical for survival of the cells. If cooling is too rapid, the cell can not loose water, will become highly supercooled, and then at some temperature below -10°C will freeze intracellularly.

Ice is formed initially in the external medium and as dehydration continues the internal solute concentration of cells increases progressivelly the small amount of solution between the ice crystals becomes also more concentrated and its freezing point is depressed again. The cells of the embryo respond to this solution of higher than normal concentration by loosing more water. If cooling continues sufficiently slow ice crystals grow, the solution surrounding them becomes more concentrated, and the cells continue their dehydration. In this way, most of the intracellular water can be removed and formation of intracellular ice is prevented. However, this is not always beneficial for the cells and intracellular ice formation occurs when the freezing rate is increased rapidly and the embryos are plunged in liquid nitrogen.

Since the dehydration process can not begin until ice forms, containers are seeded to initiate ice formation so that dehydration can occur. We then prevent supercooling by seeding or inducing ice formation. This is performed by touching the end of the straw with cold forcepts that have previously been inmersed in liquid nitrogen.

Cooling rates: The correlation between the cooling rates that lead to the formation of intracellular ice and the cooling rates that leed to cell death have been studied (17). The correspondence between the formation of intracellular ice in a cell and the death of the cell is very close. Curves of survival versus cooling rates are usually inverted U's, i.e., survivals are low at low cooling rates, raise progressively with increasing cooling rates to reach a maximum, and then drop with a further increase in cooling rates. AT low cooling rates the probability of intracellular freezing is very low but cells may be killed by "solution effects" (high intracellular concentration of solutes). At high cooling rates, the probability of intracellular ice is very high. The optimum rate is a rate low enough to minimize the probability of the formation of appreciable intracellular ice and high enough to minimize injury from solution effects. The limiting factor is the rate of passage of the water through the cell membrances which depends of a number of factors such as indivual cell permeability to water, the surface to volume ratio, temperature, and concentration gradient between the two sides of the cell.

In early studies, the embryos were cooled slowly to very low temperatures (-6-°C or to -100°C) and then were also thawed slowly. Today, the techniques for cryopreservation of the embryos have changed and faster cooling and thawing rates are geing used Lehn-Jensen (13) cooled embryos to -30°C at 0.3°C/min and then plunged into liquid nitrogen. He cooled another group of embryos to -25°C at 1°C/min before plunging in liquid nitrogen and obtained 57 and 44 % pregnancy rates respectively. In general, embryos are cooled to seeding temperatures (-5°C) at rates of 2°C to 4°C/min, held at that temperature for 10 to 15 minutes and seeded during that period, cooled at 0.3°C to 0.5°C/min to -30°C, and then plunged in liquid nitrogen.

The survival of the embryo no only depends on the rate of freezing but also on the thawing rate. Embryos that are cooled at slow rates need to be thawed slowly and those frozen at faster rates need to be thawed faster. There is not a standard temperature to thaw them. Embryos that are frozen at rates between 0.3 and 0.5°C/min. and plunged in liquid nitrogen between -30 and -38°C are thawed in water baths with temperatures ranging from 22 to 37°C. The critical points during thawing seem to be the time taken to transfer the straw from the liquid nitrogen to the water bath and the time taken to thaw the straw.

In summary, the techniques for long term preservation of bovine embryos were reviewed with emphasis in the basic components present in flushing and incubation media, principles of cryopreservation, mechanism of action of cryoprotectants, and theoreies of ice formation. Bovine embryos are very sensitive to cooling and thawing rates. The earlier methods for freezing embryos consisted of cooing them at very slow rates to -60°C and then plunge in liquid nitrogen. That method of cooling required slow warming rates. Today, cooling rates from seeding temperature (-5°C to -7°C) to -30°C or -35°C may range between 0.2 and 0.5°C/min. Embryos frozen at this fast rate may be thawed at faster rates in water baths at 30°C to 37°C. There are also different methods of deglycerolization and an alternate method used at Cryovatech was presented.

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