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Cryopreservation of oocytes and embryos: methods and applications

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ABSTRACT

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The benefits of oocyte and embryo cryopreservation originate from its ability to arrest all biological processes and place cells into a state of suspended animation. Such interruption of embryogenesis for any period of time provides a powerful method of controlling animal reproduction. The universal appeal of this approach is illustrated by recent surveys of bovine embryo transfer practitioners suggesting that over one-third of embryos collected in the USA and Europe were cryopreserved. This is an area of rapid scientific and technological advancement based on rapidly evolving concepts in the fields of cryobiology, embryology and animal reproduction.

INTRODUCTION

Over the past three decades, the cryopreservation of embryos and oocytes has become a useful tool for interrupting and controlling reproductive cyclicity. Most research and application has concentrated on three species: mouse, cattle and human. Mouse embryos are used for basic and applied research on cryobiological mechanisms. Applications include banks of embryos from genetically unique laboratory mice to ensure continued availability of infrequently used strains. For example, Jackson Laboratory in Bar Harbor, Maine maintains 272 strains as cryopreserved embryos. Cryopreservation is an integral component of the cattle embryo transfer industry and is used to transport genetically superior breeds internationally. Human embryos are frequently cryopreserved as an adjunct to in vitro fertilization and other assisted conception techniques for the treatment of infertility.

Since the first report of normal offspring from cryopreserved mouse embryos (Whittingham et al., 1972), similar success has been reported for 13

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other mammalian species. These include cattle, sheep, goat, pig, horse, rat, rabbit, cat, eland, baboon, marmoset, macaque monkey and human (see Rall, 1992, for references). Although tens of thousands of live offspring have been born from cryopreserved mouse and cattle embryos, the number of live offspring produced from cryopreserved embryos of other species is much lower.

Basic and applied research over the past 20 years has resulted in two approaches for embryo cryopreservation. The first, 'conventional slow freezing', evolved from research on the effects of cooling and warming rate on the survival of mammalian cells (Mazur et al., 1972). The second approach, 'vitrification', was proposed by Luyet (1937) and successfully applied recently (Rall, 1987). Both require careful control of the osmotic volume of embryos during each step of the process for success (Rall, 1992). This review will describe current methods for embryo and oocyte cryopreservation and discuss the cryobiological consequences of conventional slow freezing and vitrification procedures.

EMBRYO CRYOPRESERVATION PROCEDURES

Basic studies of cryoprotection and cryoinjury indicate that certain cellular properties of embryos, such as size and shape, permeability to water and cryoprotectant and other physiological considerations (e.g. embryo quality, cold shock and toxic sensitivities), determine the appropriate conditions for successful cryopreservation (reviewed by Leibo, 1986; Niemann, 1991). Because these cellular properties often vary depending on the species and embryonic stage, the steps and conditions of cryopreservation must be adjusted to minimize injury and optimize survival for the embryo in question.

Conventional slow freezing

Successful conventional slow freezing procedures have three distinctive features: (1) the addition of 1–2 M concentrations of glycerol or another cryoprotectant to the embryo suspension; (2) controlled freezing of the suspension during cooling to the storage temperature; (3) a characteristic sequence of changes in the osmotic volume of the blastomeres during the cryopreservation process (Fig. 1). The conventional slow freezing procedure used in our laboratory for eight-cell mouse embryos and Day 7 bovine embryos and the osmotic consequence of each step are described below.

(1) Embryos are transferred into a solution of 1.5 M glycerol in PB1 (Whittingham et al., 1972) for 20 min at room temperature (20–25°C). This step produces a transient shrink–swell change in the volume of the embryos as the cryoprotectant permeates into the cells (Fig. 1). We use 0.25 ml plastic insemination straws as the freezing container and prepare them for the one-

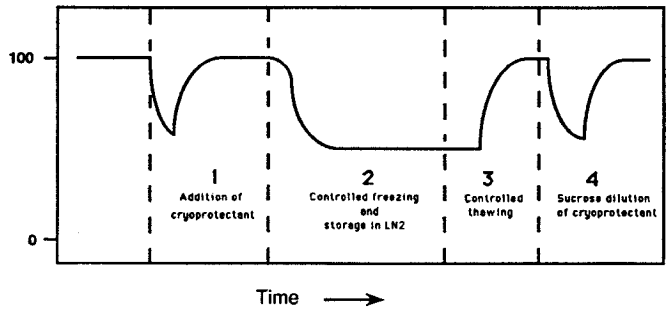

Fig. 1. Diagrammatic representation of the embryo volume changes during each step of successful conventional slow freezing. See text for details. (Figure reprinted from Rall (1992) with modifications.)

step dilution method (Leibo, 1984). Briefly, a column (6.8 cm) of 1 M sucrose in PB1 is aspirated into the straw and followed sequentially with air (0.8 cm), the cryoprotectant solution (0.8 cm), air (0.8 cm), cryoprotectant solution (1 cm) and finally air until the first column contacts and wets the cotton plug. Bovine embryos are aspirated into the straw with the final cryoprotectant column, whereas mouse embryos are pipetted into a prepared straw. Finally, the straw is heat-sealed at both ends.

(2) Straws are placed in an ethanol bath precooled to $-7°C$ and the sucrose column is seeded (see Leibo, 1984). The straw is held at $-7°C$ for 10 min to allow ice to grow into the column containing the embryos. Then the bath is cooled at 0.4 or $0.4°C\ min^{-1}$ (respectively, bovine and mouse) to $-40°C$. After 10 min at $-40°C$, straws are transferred into liquid nitrogen for storage. Seeding and controlled freezing at the optimum rate results in a progressive shrinkage of embryos due to the exosmosis of water from their cytoplasm. No change in the volume of cells occurs during rapid cooling or storage (Fig. 1).

(3) At the end of the storage period, straws are warmed in room temperature air for 10 s and then immersed in water (20°C) for 10 s. Rapid thawing reduces the osmolality of the extracellular solution as the ice crystals melt. Cells swell as water moves into the cytoplasm to restore osmotic equilibrium (Fig. 1).

(4) Immediately after thawing, the straw is shaken to mix the sucrose and cryoprotectant columns and then incubated in 35°C water for 3 min followed by 20°C water for 2–5 min. Cells progressively shrink as the glycerol leaves due to the presence of impermeable sucrose (Fig. 1). Finally, the embryos are recovered from the straw and rehydrated in PB1. The removal of sucrose from the suspending solution results in swelling to normal isotonic volume (Leibo, 1984).

Vitrification

Successful vitrification procedures have three distinctive features: (1) no ice forms in the embryo suspension during cooling, storage or warming; (2) cells are osmotically dehydrated prior to cooling by controlled equilibration in a highly concentrated solution of cryoprotectants (greater than 6 M); (3) a characteristic sequence of changes occurs in the osmotic volume of embryos during the cryopreservation process (Fig. 2). Vitrification offers considerable promise for simplifying and improving the cryopreservation of cells because controlled-rate freezing equipment is not required and potential injury associated with the formation of ice in the suspension is eliminated (Rall, 1987). Vitrification has been successfully applied to several species of mammalian embryos, including mouse, cattle, sheep, goat, rabbit and rat (Rall, 1992). The vitrification procedure used in our laboratory for eight-cell mouse embryos, Day 6 sheep and Day 7 bovine embryos and the osmotic consequence is described below.

(1) Embryos are washed in PB1 containing 6% bovine serum albumin (BSA) and transferred into a solution of 1.6 M glycerol and 6% BSA in PB1 for 20 min at room temperature. Embryos are then rinsed in a solution of 4.2 M glycerol and 6% BSA in PB1 for 1 min and placed into the final vitrification solution (VS3a; 6.5 M glycerol and 6% BSA in PB1). Embryos undergo a series of osmotic changes in their volume during the equilibration steps (Fig. 2). First, embryos undergo a transient shrink–swell change in volume in 1.6 M glycerol and then shrink in the more concentrated glycerol solutions. We use plastic insemination straws as the vitrification container and prepare them for the one-step dilution method. Briefly, a column (7.5 cm) of 1 M sucrose in PB1 is placed into the straw using a syringe and hypodermic needle. Then a 1 cm column of solution VS3a is placed adjacent to the sucrose column but separated by a 0.5 cm air space with dry walls. Mouse and bovine

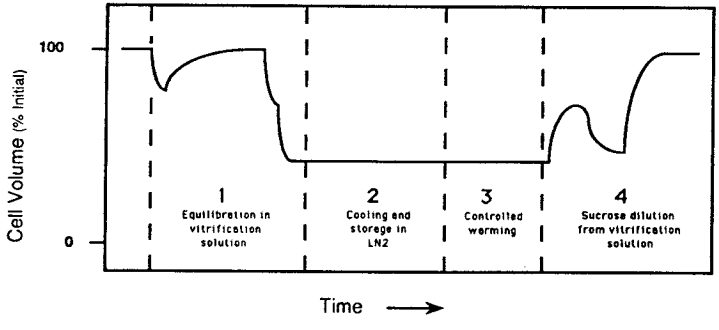

Fig. 2. Diagrammatic representation of the embryo volume changes during each step of successful vitrification. See text for details. (Figure reprinted from Rall (1992) with modifications.)

embryos are pipetted into the vitrification solution column. Finally, the straw is heat-sealed at both ends.

(2) One minute after embryos are transferred into solution VS3a, the straws are cooled by transfer into −170°C nitrogen vapor (cooling rate: 200°C min^{-1}). The entire suspensions vitrifies (i.e. becomes a glass) at about −120°C. Straws are stored ion liquid nitrogen. There are no changes in the volume of embryos due to cooling or storage (Fig. 2).

(3) At the end of the storage period, straws are warmed in 20–25°C air for 10 s and then immersed in water (20°C) for 10 s. Rapid warming results in softening of the glassy suspension into a liquid. No change in the volume of cells occurs during warming (Fig. 2).

(4) Immediately after warming, the straw is shaken to mix the sucrose and cryoprotectant columns and then incubated in 35°C water for 3 min followed by 20°C water for 2–5 min. Embryos initially swell when mixed with the sucrose diluent and then progressively shrink as the glycerol leaves the cytoplasm. Finally, the embryos are recovered form the straw and rehydrated in PB1. The removal of sucrose from the suspending solution results in swelling to normal isotonic volume (Fig. 2).

Degrees of embryo cryopreservation success

Both cryopreservation protocols yield similarly high rates of embryo survival. For example, a high proportion of eight-cell mouse embryos develop in vitro following conventional slow freezing (90% of 486 embryos) (Rall et al., 1986) or vitrification (86% of 123 embryos). Furthermore, eight-cell mouse embryos cryopreserved by either method exhibit high rates of development in vivo after embryo transfer. In one study (W.F. Rall and M.J. Wood, unpublished results), 75% of 157 frozen embryos and 64% of 194 vitrified embryos developed into late-stage fetuses and live-born pups after transfer to foster mothers.

Similar results have been reported when the same cryopreservation protocols are applied to bovine embryos. For example, a high proportion of Day 7 bovine embryos developed in vitro following conventional slow freezing (65% of 226 embryos) (Leibo, 1988) or vitrification (66% of 71 embryos). Approximately 42% of 476 frozen bovine embryos (Leibo, 1986) and 47% of 17 vitrified bovine embryos established pregnancies after thawing and embryo transfer. These results indicate that the protocols listed above yield comparable pregnancy rates and an efficiency of about 80% when compared with the success of contemporaneous fresh embryo transfer (Leibo, 1986).

Many alternative vitrification solutions and procedures have reported similar success (e.g. Massip et al., 1986). A recent report of an ethylene glycol-based vitrification solution may be especially appropriate for early cleavage-stage embryos that exhibit limited permeability to glycerol (Kasai et al., 1990).

OOCYTE CRYOPRESERVATION

Despite considerable research during the past 15 years, only modest progress has been made in developing oocyte cryopreservation (Parks and Ruffing, 1992). The greatest success has been obtained with mature mouse oocytes. Although over 100 late-stage fetuses or normal offspring have been produced from cryopreserved mouse oocytes following IVF and embryo transfer, the overall rate of development is too low for most applications. For example, only 6–14% of conventionally frozen oocytes (Whittingham, 1977; Glenister et al., 1987; Schroeder et al., 1990) and 20–38% of vitrified oocytes (Nakagata, 1989; Kono et al., 1991; Wood et al., 1991) produced fetuses or offspring. This corresponds to an overall efficiency of less than 65% when compared with untreated oocytes. Very little progress has been made in cryopreserving domestic animal oocytes. Live late-stage fetuses and young have been reported only from cryopreserved rabbit oocytes, but the overall survival rate was low (2–6%) (Al-Hasani et al., 1989; Vincent et al., 1989). Cattle oocytes exhibit low fertilization rates in vitro after slow freezing and few (0–13%) develop to the two-cell stage (Schellander et al., 1988; Lim et al., 1991).

Special cytological features of mature oocytes, namely the meiotic spindle, cortical granules and cytoskeleton, are thought to be susceptible to damage during cooling and exposure to cryoprotectants. The simple process of cooling mouse (Pickering and Johnson, 1987; Glenister et al., 1987), sheep (Moor and Crosby, 1985), and cattle (Richardson and Parks, 1992) oocytes to 25 or 4°C results in depolymerization of spindle microtubules. Although spindles reassemble when mouse oocytes are warmed and incubated at 37°C for 60 min, the potential for genetic anomalies due to errors in chromosome movements are a major concern. However, a recent report indicates that at least one consequence of meiotic spindle alterations, aneuploidy, does not increase after cryopreservation (Bos-Mikich and Whittingham, 1991). Exposure to dimethylsulfoxide also results in reversible spindle disassembly and the formation of microtubular asters at the spindle poles of mouse (Johnson and Pickering, 1987) and rabbit (Vincent et al., 1989) oocytes.

Oocyte cryopreservation is also reported to produce structural changes in the zona pellucida, called 'zona hardening', that reduce penetration of spermatozoa and fertilization (Johnson et al., 1988). However, Wood et al. (1989) report that zona hardening can be reduced or eliminated by adding serum to the cryoprotectant solution. Other incompletely defined effects of cooling that result in alterations to lipid droplets and/or cell membranes have been reported (Didion et al., 1990).

Recent research suggests several novel approaches for improving the success of oocyte cryopreservation. First, Carroll et al. (1990) report that isolated primary follicles of the mouse can be cryopreserved, matured in vivo

and yield normal young following IVF. Although technical difficulties during in vivo maturation currently limit the overall efficiency (3%), this approach may provide large numbers of mature oocytes for IVF or other purposes. Second, the addition of antifreeze glycoproteins from antarctic fish to a vitrification solution has been reported to protect immature pig oocytes from chilling injury (Rubinski et al., 1991). About 25% of vitrified pig oocytes matured in vitro after warming. This result indicates that membrane-associated chilling injury can be reduced by a new class of extracellular cryoprotectants and ultrarapid cooling procedures.

CONCLUSIONS

The application of fundamental cryobiological concepts yield two different approaches that satisfy the requirements for successful embryo cryopreservation, conventional slow freezing and vitrification. Despite radical procedural differences, both approaches seek common goals: the osmotic dehydration of cells prior to storage in liquid nitrogen and prevention of the deleterious effects of chemical toxicity and intracellular freezing. Successful cryopreservation usually requires optimization of each step of the procedure to account for the size, permeability and physiological characteristics of the constituent cells. Simple, effective cryopreservation protocols have been developed for embryos of several mammalian species, e.g. mouse and cattle. However, the intrinsic characteristics of mammalian oocytes and some mammalian embryos (e.g. pig) limit effective application of current procedures. Systematic studies of the molecular, cellular and physiological basis of injury associated with cooling and cryoprotectants are urgently needed. Promising areas of research include the cryopreservation of primary oocytes prior to the formation of fragile cytoplasmic features and the development of new cryoprotectants and additives that stabilize fragile cellular components.

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