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Several aspects of animal embryo cryopreservation: anti-freeze protein (AFP) as a potential cryoprotectant

A.V. Makarevich[1,2], *E. Kubovičová*[2], *M. Popelková*[3], *D. Fabian*[5], *Š. Čikoš*[5], *J. Pivko*[2] *and P. Chrenek*[2,4]

Animal Production Research Centre (APRC) Nitra; Centre for Assisted Reproduction, Paul Joseph Šafarik University, Košice; Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture, Nitra; and Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic

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Summary

With the development of embryo technologies, such as *in vitro* fertilization, cloning and transgenesis, cryopreservation of mammalian gametes and embryos has acquired a particular interest. Despite a certain success, various cryopreservation techniques often cause significant morphological and biochemical alterations, which lead to the disruption of cell organelles, cytoskeleton damages, cell death and loss of embryo viability. Ultrastructural studies confirm high sensitivity of the cell membrane and organelle membrane to freezing and thawing. It was found that many substances with low molecular weights have a protective action against cold-induced damage. In this concern, an anti-freeze protein (AFP) and anti-freeze glycoproteins (AFGPs), which occur at extremely high concentrations in fish that live in Arctic waters and protect them against freezing, may be of potential interest for cryostorage of animal embryos at ultra-low temperatures. This mini-review briefly describes several models of AFP/AFGP action to preserve cells against chilling-induced damages and indicates several ways to improve post-thaw developmental potential of the embryo.

Keywords: Anti-freeze glycoprotein, Anti-freeze protein, Cryoprotectant, Embryo, Freezing, Vitrification

Introduction

Embryo cryopreservation has become an essential component of artificial reproductive technologies enabling storage of valuable germplasm produced at *in vitro* fertilization programme (*in vitro* embryo production), intracytoplasmic sperm injection–ICSI, transgenesis and cloning. Embryo cryopreservation also provides a means to store excessive embryos when the yield from superovulation is larger than the number of available recipients. Moreover, this approach is effective to preserve embryos from exotic, rare or endangered species, to protect valuable animal lines from potential loss due to environmental disasters, genetic drift and infectious diseases (Landel, 2005; Moore & Bonilla, 2006).

There are six steps to successful embryo cryopreservation: exposure to cryoprotectant; cooling to subzero temperatures; storage; thawing or warming; removal of cryoprotectant; and return to a physiological environment (Liebermann *et al.*, 2003). At present two techniques, conventional slow cooling/freezing and vitrification, are used. The time required to freeze embryos using the classical technique of slow freezing varies from 90 min to 5 h, depending upon freezing protocol. Freezing involves precipitation of water to form ice, which causes separation of water from diluted substances. The resulting high concentration of diluted substances, as well as intracellular ice formation, may damage the cell. Despite the balance between these two factors during slow freezing, ice formation, osmotic and cold influence may cause cracking of the *zona*

[1]All correspondence to: A.V. Makarevich. Animal Production Research Centre (APRC) Nitra, 95141 Luzianky near Nitra, Slovak Republic. Tel: +421 37 6546 335. Fax: +421 37 6546 480. e-mail: makarevic@cvzv.sk
[2]Animal Production Research Centre (APRC) Nitra, Slovak Republic.[6]
[3]Centre for Assisted Reproduction, Paul Joseph Šafarik University, Košice, Slovak Republic.
[4]Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture, Nitra, Slovak Republic.
[5]Institute of Animal Physiology, Slovak Academy of Sciences, Košice, Slovak Republic.
[6]APRC Nitra is a member of the Centre of Excellence 'Biomembranes' (APVV VVCE 0064-07-05).

pellucida, disintegration of blastomeres and alterations in cytoskeleton.

Numerous studies have been directed to reduce freezing times and eliminate the expensive accessories required for conventional slow freezing. One of the ways to avoid damage caused by ice crystals is the use of the vitrification technique. This freezing method is an alternative to classical freezing with equilibration. This method was first described in 1985 with mouse embryos (Rall & Fahy, 1985) and 8 years later it was successfully repeated by Ali & Shelton (1993). Martino *et al.* (1996) indicated that bovine oocytes vitrified at high-rate cooling were able to develop to blastocysts. Successful vitrification of bovine *in vitro* produced embryos was described following the invention of the 'open-pulled straws' (OPS) method by Vajta *et al.* (1997). Using this technique, live offspring have been produced consistently from cryopreserved porcine blastocysts (Berthelot *et al.*, 2000; Beebe *et al.*, 2002). Rabbit embryos vitrified by this method showed a dramatic decrease in post-thaw hatching rate (Popelkova *et al.*, 2005). Following progress in assisted reproduction technology in 1999–2000, vitrification protocols were also successfully applied to freeze human oocytes (Kuleshova *et al.*, 1999; Yoon *et al.*, 2000). Although this method has existed for about three decades it still produces variable results. Obviously, success is dependent upon several factors that include embryo stage and quality, embryo species and derivation, cooling and warming rates, culture conditions and others. Efforts to optimize these conditions will further enhance survival and future developmental potential of the embryo.

Factors affecting cryopreservation outcomes

The factors that cause stage-specific sensitivity to cold shock in embryos are unknown, although ultrastructural changes in cell structure have been described (Palasz *et al.*, 1997; Vajta *et al.*, 1997; Fair *et al.*, 2001; Visintin *et al.*, 2002; Cocero *et al.*, 2002; Pivko *et al.*, 2003; Popelkova *et al.*, 2005). Based on previous knowledge and the study of Vajta *et al.* (1997), it is possible to predict the existence of mechanisms at 24 h after vitrification that leads to the repair of cellular structures, for example intercellular junctions, that are responsible for compaction of the morula and formation of the blastocyst.

Some of the factors that affect the extent of cellular damages and survival of frozen embryos are: the type of the medium; cryoprotectant; rate of cooling and temperature level at ice crystal formation; duration of cryopreservation procedure; species; developmental stage of the embryo; the system of embryo production; and preparation of recipients (Fabian *et al.*, 2005). In particular, rabbit embryos at the morula stage (Silvestre *et al.*, 2003; Naik *et al.*, 2005; Papis *et al.*, 2005), blastocysts (Lopez-Bejar & Lopez-Gatius, 2002) and zona-free expanded or hatching blastocysts (Cervera & Garcia-Ximenez, 2003; Popelková *et al.*, 2008) were cryopreserved; higher rates of development were achieved when blastocyst-stage embryos were used.

The possible cause of reduced embryo viability during freezing–thawing is the disruption of cell organelles, in particular the cytoskeleton, as a result of intracellular ice formation (Dobrinsky, 1996). It has been demonstrated that cryopreservation of equine (Tharasanit *et al.* 2005) or rabbit (Makarevich *et al.*, 2008) embryos leads to actin cytoskeleton disruption and cell death. The viability of frozen–thawed porcine embryos was improved when cytoskeletal damage was reduced after addition of the actin polymerization inhibitor cytochalasin B (Dobrinsky *et al.*, 2000). Modification of the actin cytoskeleton and injury to other organelles may affect signal transduction and lead to programmed cell death or apoptosis. Cryopreservation was found to cause an increase in the apoptotic rate in post-thaw bovine (Baguisi *et al.*, 1999; Marquez-Alvarado *et al.*, 2004; Park *et al.*, 2006), porcine (Fabian *et al.*, 2005) and mouse (Ahn *et al.*, 2002) embryos.

Survival rate after cryopreservation may also be decreased in micromanipulated embryos (Popelkova *et al.*, 2005; Makarevich *et al.*, 2008) due to their high sensitivity to external influences, when compared with intact embryos. Popelkova *et al.* (2005) described severe degenerative alterations in cellular organelles, detected on the ultrastructural level, in rabbit gene-microinjected OPS-vitrified embryos when the cryoprotectants ethylene glycol (EG) and dimethylsulfoxide (DMSO) were used. The damage was attributed not to the microinjection procedure itself but rather to a direct influence of the vitrification procedure. Although this vitrification protocol was used successfully on bovine embryos (Vajta *et al.*, 1997, 1998), it was found that rabbit embryos vitrified by this protocol showed a dramatic decrease in post-thaw hatching rate (Popelkova *et al.*, 2005).

A vitrification technique that used ethylene glycol, Ficoll 70 and sucrose in the vitrification medium (EFS solution) was initially described by Kasai *et al.* (1992) and Gajda (1996) and was modified by Papis *et al.* (2005). Results for this vitrification technique showed that most warmed rabbit embryos survived post-thaw, 71% of embryos developed to the blastocyst stage and 23.5% were developed to term (Papis *et al.*, 2005). Subsequently this technique was successfully used for the cryopreservation of rabbit gene-microinjected embryos (Makarevich *et al.*, 2008).

Cryoprotectants, sugars and macromolecules

The most commonly used medium for vitrification is phosphate-buffered saline (PBS). Cryoprotective substances are necessary for successful cryostorage of living cells. Cryoprotectants are divided into two groups:

(i) penetrating: glycerol, ethylene glycol, DMSO, propanediol;
(ii) non-penetrating: saccharides, proteins and polymers (Ficoll, polyethylene glycol [PEG]).

The main components of vitrification solutions are cell membrane-penetrating substances. These components are hydrophilic with a strong dehydrating action and have the ability to reduce the freezing point of solution, from −10 °C to −35 °C or even up to −45 °C, and to delay spontaneous ice formation. A freezing point is dependent upon cryoprotectant concentration as well as on the speed of freezing. At slow freezing the cells have a longer time for rehydration. Cell membrane-penetrating cryoprotectant is able to bind with intracellular water, therefore the water withdraws from the cell very slowly. Therefore, the critical intracellular concentration of minerals is reached at lower values.

Test results showed that ethylene glycol and then glycerol were the least toxic of several cryoprotective substances. In particular, ethylene glycol appears to have a low toxic effect on mouse embryos (Ali & Shelton, 1993) and a rapid diffusion coupled with a quick equilibration of ethylene glycol into the cell through the *zona pellucida* and the cellular membrane (Emiliani *et al.*, 2000). From this reason ethylene glycol is more often used for the vitrification. Generally, the best cryoprotectants are those that penetrate cells faster, because they reduce exposure time and minimize cell shrinkage. Moreover, these substances are able diffuse more quickly from the cells; the cells will return to their initial volume faster and will be protected from osmotic injury.

It was found that many substances with low molecular weight have protective actions against cold-induced damage. Therefore, many researchers have compared the action of various cryoprotective agents on mammalian embryos. Hasler *et al.* (1997) in their extensive study reported both the development of 68% embryos to hatching following the freezing of blastocysts in 1.4 M glycerol or a rate of 62% of embryos after freezing in 1.5 M ethylene glycol.

High-molecular-weight additives, such as disaccharides (sucrose, trehalose and raffinose), do not penetrate across cell membranes but substantially decrease the concentration of intracellular cryoprotectants needed for successful cryostorage and therefore minimize their toxic effect. Saccharide solution may also serve as an osmotic buffer during thawing, when cryoprotectant is toxic to the cells. On thawing, water enters the cell faster than the cryoprotectant diffuses out, therefore fast flushing the vitrification solution out of the cells is necessary. Excessive water flow may lead to damage due to osmotic swelling. Sucrose acts as an osmotic buffer by reducing osmotic shock. Sucrose at a high concentration (i.e. 1 mol/l) is not toxic to embryos or oocytes (Kuleshova *et al.*, 1999); sucrose cannot completely preserve the cells against swelling but it can reduce the speed and extent (Lieberman *et al.*, 2003). Injection of trehalose into the oocyte's cytoplasm has been shown to be a promising approach and results in increased survival of oocytes after thawing (Eroglu *et al.*, 2002). Trehalose rapidly exits the cytoplasm of the developing embryo and does not affect developmental capacity negatively (Eroglu *et al.*, 2005).

Macromolecules, such as PEG, polyvinylpyrrolidone (PVP), Ficoll, dextran and polyvinyl alcohol, modify the properties of the vitrification solution (Asada *et al.*, 2002). These polymers are generally of low toxicity and protect the embryos against cryodamage by reducing mechanical stress; they affect the viscosity of the vitrification solutions and lower the toxicity of cryoprotectants by reducing the concentration required to reach vitrification itself. These polymers form viscous matter enclosed the embryo and also prevent crystallization during vitrification and warming. The most commonly used polymer among those mentioned above is Ficoll, mainly in combination with ethylene glycol and sucrose (Kasai *et al.*, 1990).

Various protein additives, including egg yolk, were also tested but were not implemented for wide use due to the resulting optically dense appearance and difficulties in microscopical manipulations. Particularly in cattle, serum additives, bovine serum albumin (BSA)-based preparations, recombinant BSA and hyaluronan are widely used supplements for vitrification media (Lane *et al.*, 2003).

Many authors (Bautista & Kanagava, 1998; Sommerfeldt & Niemann, 1999) have reported that embryos are highly permeable following the use of cryoprotective agents (particularly ethylene glycol) that are not toxic at various concentrations. Freezing media may contain proteins, serum or other biological macromolecules or synthetic ones such as PEG, polyvinylpyrrolidone or Ficoll. One of the disadvantages of naturally derived macromolecules is that they act as carriers for many substances such as steroids, fatty acids, citrates. Other macromolecules, such as hyaluronan or glucosaminoglycans, are also used as serum substituents for embryo freezing (Palasz *et al.*, 1993) and several of these are used for embryo culture.

Anti-freeze protein

It has been known for the past three decades that fish that live in Arctic waters have developed mechanisms

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that protect themselves against freezing. Scholander *et al.* (1957) found that these fish contain a factor in their blood that prevents freezing. De Vries & Wohlschlag (1969) found that this factor is a protein that occurs at extremely high concentrations (35 g/l). These are proteins called anti-freeze proteins (AFPs) and anti-freeze glycoproteins (AFGPs) and are present in tissues and blood of fish (Yeh & Feeney, 1996). Anti-freeze proteins have been found not only in animals but also in plants. Four groups of these proteins exist, one of which is glycosylated (Yeh & Feeney, 1996). Glycosylated protein or AFGPs consists of repeating tripeptides Ala–Ala–Thr with a disaccharide on each Thr. To date eight fractions of AFGPs have been identified, all have molecular weight in the range 2.6–33.7 kDa. Fractions 7 and 8 are the smallest and contain a Pro that follows Thr and represents about 75% of the total AFPG in the serum. AFP type I consists of 60% alanine with helical structures of 4.3 and 3.3 kDa in size. Type II is rich in cysteine and is 14 kDa. Type III has only 66 amino acids and is rich neither in alanine nor in cysteine.

Recently, AFP function was associated with freezing only, as it was known that AFPs can decrease the freezing temperature of solutions, inhibit recrystallization during thawing or bound to an ice nucleator (Yeh & Feeney, 1996). In the 1990s, Rubinsky *et al.* (1990) and Arav *et al.* (1994) discovered a new property of these proteins when working with porcine oocytes, which are very susceptible to cold damage. When they put the oocytes in the presence of AFGPs in the refrigerator the oocytes were still intact the following day and after rewarming they appeared normal. Further studies showed that oocytes, if cooled to 4 °C without the addition of AFGPs, were unable to retain a normal electric membrane potential after thawing. However, when AFGP was added before cooling, 6.5% of rewarmed cells exhibited an electric potential that was comparable with that of fresh oocytes (Rubinski *et al.*, 1990). The mechanism of cell protection was described 2 years later. Using patch-clamp technique on porcine granulosa cells it was found that a low concentration of AFP (0.5 mg/ml) blocks K^+ and Ca^{2+} channels during cooling, whereas other proteins, such as BSA or soybean trypsin inhibitor, do not block these ion channels (Arav *et al.*, 1994).

After these primary results several controversial reports appeared in the literature. For example, AFPGs did not increase the vitality of rat heart after hypothermia or freezing (Wang *et al.*, 1994). Rat sperm, which are known to be sensitive to cooling, were not stabilized following cooling to 5 °C and subsequent warming even in the presence of AFPs. Thylakoid membranes were stabilized after the addition of AFPs and AFGP during freezing and also when stored at 0 °C for 7 days (Hincha *et al.*, 1993). The many unresolved aspects that arose from these findings were explained after finding the mechanism for AFP action.

Mechanisms of AFP/AFGP action

Several molecular models for the mechanism of AFP and ice binding were reported (see review by Madura *et al.*, 2000). From these schemes it is obvious, that the specific forces involved in the AFP interaction with ice are still not fully known. However it is clear that the specific ability of AFPs to bind to ice is the property that distinguishes AFPs from non-AFPs. This property is responsible for the differences observed in the levels of AFP activity.

Compared with many other solutions, AFPs suppress the temperature of ice crystal formation kinetically thus preventing thermal shock. This property enables fish to survive in water temperatures lower than the freezing point of their blood or other body fluids by modifying or preventing ice crystal growth. This process protects cell membranes against cold-induced injury (Yeh & Feeney, 1996; Madura *et al.*, 2000). These ubiquitous properties are interesting as a potential medical application, as well as in areas in which long-term storage of biological material is needed (Fletcher *et al.*, 1999).

Rubinski *et al.* (1990) showed that AFPs protect cell membranes following hypothermic stress. They found that bovine and porcine oocytes maintained their membrane potentials after chilling to 4 °C and warming in the presence of AFPs. This protection was due to the interaction between anti-freeze peptides and integral cell membrane proteins. In an attempt to understand the different properties of AFGPs, a series of studies was performed on liposomes as a model for the effects of lipid-phase transitions. It was concluded that the stabilizing effects of AFGPs on intact cells during chilling, reported in earlier studies (Shaw *et al.*, 2000; Sugimoto *et al.*, 1996), were perhaps due to non-specific effects on the lipid components of native membranes (Lee *et al.*, 2000; Kagabu *et al.*, 2000).

Use of AFPs for cryostorage of animal embryos

AFPs were mostly used as cryoprotective agents in fish embryo cryopreservation. Anti-freeze protein significantly improved chilling resistance at 0 °C, particularly in 2-cell microinjected zebrafish embryos (Robles *et al.*, 2007). The authors stated that AFPs protect cellular structures by stabilizing cellular membranes. To improve cryoprotection of the cellular compartment of zebrafish embryos, AFP type I and AFP type III were incorporated into the embryo either by microinjection (Robles *et al.*, 2007), laser pulse (Kohli *et al.*, 2007) or by a non-invasive method of embryo incubation in medium containing AFPs (Martinez-Paramo *et al.*, 2008).

In mammalian embryos, AFPs were tested for cryopreservation of equine embryos either given alone or together with glycerol in medium; no difference was observed between both groups (Lagneaux *et al.*, 1997). Sheep embryos that had been cooled and stored at 4 °C in the presence of AFPs, showed similar embryo survival and pregnancy rates as fresh embryos (Baguisi *et al.*, 1997).

Available reports on AFP usage in mammalian embryo preservation mainly describe its use for the storage in chilling conditions (0–4 °C), these proteins have, as yet, no application for ultra-low freezing (−196 °C, in liquid nitrogen). In our opinion, this application is worthy of attention from researchers who are investigate AFPs' cryoprotective properties. AFP is a potential candidate as a cryoprotective substance.

Ultrastructural morphology of cryopreserved cells

Although huge efforts have been made to improve freezing methods, numerous studies have shown destruction of cytoplasmic membrane and cell organelles in embryos frozen both by classical method or vitrification. Sensitivity to freezing in embryos of most animal species is tightly dependent on the presence of vesicular inclusions in embryoblastic cells. Bovine embryos have an abundance of such inclusions at early stages of development, and the loss of such vesicles in blastocyst cells results in decreased sensitivity to freezing (Pivko *et al.*, 1998).

Cryopreserved blastocysts usually display a collapse of the blastocoele cavity and cell swelling, a general distension or shrinkage of mitochondria and massive increase in the amount of vesicles, vacuoles and secondary lysosomes. Some embryos are able to recover, whereas the remaining degenerate (Fabian *et al.*, 2005).

The majority of organelles in early embryos are formed by membranes. The endoplasmic reticulum is an organelle formed by fine pipes and flat cisternae the walls of which form membranes. The Golgi apparatus is also formed by flat cisternae. Mitochondria are formed by external and internal membranes and cristae. Cytoplasmic membrane and other membranes of organelles are present in trophoblastic and embryoblastic cells of early embryos. Results from ultrastructural studies confirm the high sensitivity of cytoplasmic membrane and organelle membrane to the influence of freezing and thawing. It is assumed that electron microscopic analysis is required for the quality evaluation of early embryos, which are more sensitive to cold-induced damage than blastocysts but hardly ever evaluated by stereomicroscope. The three most frequently appearing ultrastructural alterations in cellular organelles and cytoplasmic structure are a waving of cytoplasmic membrane, formation of enlargements and vesicles orientated to widened space (Pivko *et al.*, 2003). Microvilli of trophoblastic cell surface have a disordered configuration with the occurrence of membrane vesicles. Detritus of damaged cell parts accumulates in the perivitelline space more often than in the blastocoele cavity. Desmosomal tight junctions between trophoblastic cells appear without changes and nexus-like junctions appear undamaged, but they are smaller and less expressed. The cytoplasm of trophoblastic and embryoblastic cells after freezing–thawing is characterized by an increased number of vacuoles and vesicles with electron-dense matter, vacuolated dark mitochondria, widened cisternae of the Golgi apparatus and granulated endoplasmatic reticulum. Lipid droplets appear undamaged, but the surrounding endoplasmatic reticulum membranes are often widened. Cell nuclei are often vacuolated and the nuclear membrane is expanded at several sites (Pivko *et al.*, 2003). Cells respond to cold shock more often due to alterations in structure of mitochondria and cytoplasmic membrane. Part of the mitochondrion appears distended, resulting in electrolucent areas in the mitochondrial matrix, whereas other areas are shrunken and display increased electron density. In many cases, the smooth endoplasmic reticulum is also swollen (Fabian *et al.*, 2005). The organelles in general and cells of cryopreserved embryos react by either distension or shrinkage, which, in the case of whole cells (at least of a proportion of them), results in degeneration. Some of these degenerated cells are extruded into the perivitelline space or blastocoele (Vajta *et al.*, 1997; Fabian *et al.*, 2005).

Several directions to improve post-thaw embryo survival

Cold shock-induced damage may be associated with changes in membrane permeability and cellular microfilament structure (Mavrides & Morroll, 2005). Therefore, the artificial stabilization of cytoskeletal microfilaments may be a tool to improve survival of post-thaw embryos. For this purpose the addition of cytochalasin B to embryos prior to vitrification was used in porcine (Dobrinsky *et al.*, 2000), mouse (Chen *et al.*, 2005) or equine (Tharasanit *et al.*, 2005) blastocysts. However, this step was useful only in porcine embryos, whereas in mouse embryos cytochalasin B provided no benefit for improving post-warming survival; in equine embryos cytochalasin B reduced post-warming cell death, but depolymerization of actin filaments was not reversed within 6 h of culture, compromising embryo survival (Tharasanit *et al.*, 2005).

The dehydration of blastocyst stage embryos at vitrification may be impaired due to a difficulty with cryoprotectants to permeate the blastocoelic cavity, therefore increasing ice crystal formation. Therefore,

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another approach to improve post-thaw embryo developmental potential may be the artificial reduction of blastocoelic fluid when osmotic shock, permeability and ice crystal formation can be reduced (Moore & Bonilla, 2006). Thus, microsuction of blastocoelic fluid prior to vitrification improved post-warming survival, implantation and pregnancy rates in human (Vanderzwalmen *et al.*, 2002; Son *et al.*, 2003) and mouse (Chen *et al.*, 2005) embryos.

Recently, Moreira da Silva & Metelo (2005) reported that vitrification significantly reduced pore size and pore number of the *zona pellucida* of bovine embryos. Moreover, pore size following vitrification (73%) was more substantially reduced than in conventionally slow cooled embryos (46%). According to above authors, this factor may inhibit nutrient exchange in culture and also the ability of embryos to hatch. Furthermore, it was observed for mouse embryos, that 0.7 M sucrose can cause zona hardening (Vincent *et al.*, 1991). Therefore, further optimalization of culture conditions and/or vitrification solution may help to solve these problems.

Vitrification has become useful for storage of micromanipulated embryos. Thus, Nguyen *et al.* (2000) demonstrated that cloned embryos were more sensitive to cryopreservation and successfully vitrified using a lower concentration of ethylene glycol. This factor may be due to the greater access of the cryoprotectant caused by hole formation in the *zona pellucida* of micromanipulated embryos. Makarevich *et al.* (2008) reported that micromanipulated transgenic rabbit embryos vitrified in vitrification medium that contained ethylene glycol and Ficoll 70 showed higher survival rates than those vitrified in medium containing ethylene glycol and DMSO. Therefore, efforts in improving cryopreservation protocols may be important to enhance the survival rate of micromanipulated embryos.

Conclusion

Cryostorage, i.e. freezing or vitrification of mammalian embryos, has a real significance in embryo production using the methods of *in vitro* fertilization, embryonal and somatic cloning, chimeric and transgenic embryo formation or the preservation of endangered animal species. In farm animals, cryostorage is reasonable in order to create a bank of embryos or spermatozoa from outstanding individuals or endangered species. In human medicine, cryopreservation of gametes is an integral part in the function of assisted reproduction centres.

Although great progress has been achieved in the area of structural characterization and properties of AFPS and AFGPs, the molecular mechanisms behind inhibiting ice growth are still not completely clear. Common properties of AFPs and AFGPs, such as an ability to alter the ice formation process, to inhibit re-crystallization and to protect cell membranes, may be used in various processes associated with low temperatures. Both AFGPs and AFPs exhibit a number of unique properties that protect biological systems *in vitro*: the ability to change the normal growth habit of ice, the capacity to inhibit re-crystallization and to protect the cell membranes. These unique abilities have been tested for potential application in medicine, biotechnology and food industry. Outcomes of these tests indicate a potential possibility to use AFPs in cryosurgery, increasing the destruction of solid tumours through mechanical damage to cells caused by bi-pyramidal ice crystals or as potential food additives that inhibit the formation of large ice crystals in frozen foods. Moreover, these AFPs may be useful in veterinary and agricultural practice.

In recent years, a certain success in embryo survival following vitrification was reached in many animal species. Nevertheless, further optimization of culture conditions for *in vitro* embryo production, both prior to cryopreservation and post-warming, as well as development of reliable vitrification protocols are essential to improve pregnancy rates and the production of viable offspring.

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