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Cryopreservation of pig embryos

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The changing global needs for food and animal products require the development of breeding strategies for maximizing genetic improvement while maintaining genetic diversity. Genetic diversity can be conserved by using separate breeding herds; however, they may be expensive to maintain and inbreeding becomes a major concern. Alternative methods are needed to preserve valuable genetic resources in a reasonable and economic manner. Embryo cryopreservation allows indefinite storage *in vitro* at subambient temperatures where metabolism and other cellular functions are greatly reduced or cease, and upon recovery from storage, normal developmental competence can be resumed. Storage and transportation require little maintenance and there is no expense in animal care and little concern about disease transmission. Although there are methods for routine cryopreservation of germplasm and embryos of most livestock species, development of this technology in the pig industry is far behind and has abated improvements in genetic potential. Pig embryos are very sensitive to hypothermic conditions, and this limits their ability to withstand many conventional methods of preservation. Much research has focused on the high lipid content of pig embryos, and its role in hypothermic sensitivity and cryosurvival. Many studies have reported the conventional freezing of pig embryos, and vitrification has shown promise of eluding the difficulties associated with cooling sensitivity and ice crystallization. Recent research suggests that the embryonic cytoskeleton is susceptible to damage during cryopreservation, and this cellular disruption may be averted by using cytoskeletal stabilizers before preservation. Embryos cryopreserved by conventional freezing and vitrification under the influence of cytoskeletal stabilization have resulted in pregnancies or live offspring from recipient females after surgical transfer. Although cryopreservation technology is less advanced in pigs than in other livestock species, promising research shows evidence that researchers are close to achieving a methodology for preserving pig embryos.

Introduction

Existing genetic diversity has been used to make vast improvements in the quality and production of food and animal products. Advances in animal biotechnology used in conjunction with traditional animal breeding programmes can significantly improve the rate of genetic progress in domestic animals. But while improvements have been made, genetic diversity is being reduced as native animal populations become less isolated and breeding programmes become more globally oriented. Therefore, evolutionary processes for maintaining genetic diversity of the past are not sufficient for the future. Variability is needed to improve populations and to allow populations to adapt to environmental changes. Desirable breeds possessing beneficial production characteristics or valuable disease resistance traits are not in any one country or region, yet need to be globally available to ensure optimal production and disease resistance. Disease transmission and other health concerns limit transport of live animals and subsequent propagation globally. Conservation of genetic resources is essential for availability of desirable genes and germplasm to meet the varying needs of the future. Banking of desirable animals for unique genetic, production and disease

resistance traits will facilitate acquisition and characterization of potentially useful germplasm, ensure genetic variation through preservation of selected stocks, and facilitate use of useful germplasm in research and industry in the future.

There are methods that adequately, but not optimally, preserve germplasm and embryos from genetically superior animals of most of livestock species except the pig. While there are methods to preserve boar spermatozoa, there has been little success until recently in preserving pig oocytes and embryos. The use of embryos in addition to spermatozoa represents an increase in the efficiency of transmitting improved genetic potential. Large numbers of pigs are transported by air freight from countries in which nucleus herds are located to countries in which new breeding units are being established (Polge, 1985). Transportation costs are extremely high, and health tests and other requirements are involved. These costs and associated constraints could be considerably reduced by shipping embryos rather than live animals.

Implementation of methodologies for long-term embryo preservation and transfer in pigs would provide a foundation for effective use of the world's best genetic resources on a global basis while modernizing pork production and enhancing genetic improvement programmes. The development of a repeatable method for the long-term preservation of pig embryos would provide numerous practical applications, including transport of maternal germplasm, rapid regeneration or expansion of new and existing lines, the ability to increase selection pressure in nucleus herds, extraction of healthy stock from diseased herds, improve quarantine conditions and provide a method for the international export and import of potential breeding stock (Polge, 1977, 1985; Dobrinsky, 1993; Niemann and Reichelt, 1993). While maintaining genetic resources through embryo banking, successful embryo cryopreservation would enhance the further development of other animal production technologies such as sperm sexing, artificial insemination, *in vitro* fertilization and non-surgical embryo transfer. With the increasing use of swine in human biomedical research, embryos from valuable genetic or transgenic lines of pigs used as organ donors in xenotransplantation could be preserved for future use. Collectively, these technologies could be instrumental in the continuous production of animals of high genetic merit capable of having a significant impact on the improvement of the world pig population and on human medicine. This review will summarize the hypothermic sensitivity of pig embryos and the current progress in pig embryo preservation and emphasize its potential impact on global swine production.

Hypothermic Sensitivity

Pig embryos can be severely altered or permanently damaged by being cooled below physiological temperatures. This chilling sensitivity appears to be dependent on the developmental stage of the embryo and on the conditions under which it develops (Pollard and Leibo, 1994). Wilmut (1972) first demonstrated the apparent sensitivity to hypothermic exposure on pig embryo development. Simply, embryos cooled *in vitro* to 15 or 20°C implanted after surgical transfer to recipients, while embryos cooled to 5 or 10°C did not implant. Polge *et al.* (1974) verified this observation, noting that 15°C was the critical temperature at which damage occurs (Polge, 1977) and that rate of cooling to the critical temperature had no effect on embryo survival (Wilmut, 1986). This sensitivity was further verified by Green *et al.* (1984). Pollard and Leibo (1994) and Leibo *et al.* (1996) observed similar survival of embryos cooled to 15°C, with 90% of pig morulae developing into blastocysts. However, only 5% developed after being cooled to 14°C, while none developed after chilling to 13°C. Pig oocytes are also sensitive to cooling (Didion *et al.*, 1990) as immature oocytes are irreversibly damaged and do not survive following chilling to 15°C or lower or after cryopreservation.

The sensitivity of pig embryos to chilling is not apparent in embryos at the morula to blastocyst stage from other domestic and laboratory species, such as cattle, sheep, rodents and rabbits (for review see Dobrinsky, 1993). However, it is well understood that boar spermatozoa are extremely sensitive to cooling and cryopreservation (Dziuk and Henshaw, 1958; Pursel *et al.*, 1973), suggesting that hypothermic sensitivity may be species specific. The addition of phospholipids to boar semen was beneficial at temperatures below 15°C but was found ineffective with pig embryos (Butler and

Roberts, 1975). The addition of fetal bovine serum (Nagashima *et al.*, 1988a) to embryo storage media helped improve the sustenance and survival of expanded and hatched pig blastocysts cooled below 15°C. Furthermore, Nagashima *et al.* (1988b) observed that pig hatched blastocysts, especially those that had been cultured from expanding blastocysts (blastocysts hatched *in vitro*), had a higher tolerance to cooling than pre-hatching stage embryos. Survival of expanding blastocysts was significantly lower than *in vitro* and *in vivo* hatched blastocysts. Normal fetal development ensued after transfer of cooled (to 11°C) *in vitro* hatched blastocysts, whereas morulae and early blastocysts did not develop. Niemann (1985) attempted to chill pig embryos below 15°C in the presence of cryoprotectants known to be effective in cryopreserving embryos of other domestic species; however, the treatments did not improve hypothermic stability. Collectively, these observations suggest that there are apparent differences in the low temperature tolerance of pig embryos at different developmental stages. This cooling intolerance might be due to the high lipid content found in early developing pig embryos before blastocyst formation (Niemann, 1985; Toner *et al.*, 1986). Perhaps a qualitative change of cells or cell plasma membranes may give rise to tolerance of low temperatures (Nagashima, 1988a,b).

Cryopreservation

Conventional freezing

The sensitivity of pig embryos to chilling appears to be correlated with its sensitivity to cryopreservation (Pollard and Leibo, 1994). Early attempts at pig embryo cryopreservation were few and inconsistent in survival. Nagashima *et al.* (1989a) reported that up to 83% of hatched blastocysts cultured *in vitro* survived freezing to –20°C in 1.5 mol dimethylsulfoxide l^{-1}(DMSO) while morulae and early blastocyst stage pig embryos did not survive cryopreservation. They concluded that development to at least the expanded blastocyst stage was essential for pig embryos to tolerate freezing. Hayashi *et al.* (1989) demonstrated that expanded or hatched blastocyst stage pig embryos survived brief cryopreservation to –35°C. After thawing 11 frozen expanded blastocysts and subsequent transfer, five live piglets were born to one recipient sow. The viability of peri-hatching blastocysts after storage in liquid nitrogen (LN_2) was confirmed. Kameyama *et al.* (1990) cooled expanded blastocysts rapidly from room temperature to seeding temperature, and then slow cooled to –35°C before plunging into LN_2. After transfer of 20 frozen–thawed embryos, two live piglets were born to a recipient sow. Kashiwazaki *et al.* (1991) used conventional slow freezing and reported the birth of four live offspring after 32 frozen–thawed embryos were transferred to a recipient female, while only 11% of the cryopreserved embryos survived *in vitro*. Fujino *et al.* (1993) tested egg-yolk (used in semen cryopreservation) as a cryoprotectant for conventional freezing of pig embryos at various stages of development. After transfer of hatched blastocysts into six recipient gilts, a single recipient delivered one live piglet. They concluded that egg-yolk did not improve the cryosurvivability of pig embryos. Further research using various methods with conventional freezing of pig embryos has shown similar *in vitro* and *in vivo* post-thaw development (Hsu *et al.*, 1990; Jung and Chang, 1990; Jung *et al.*, 1990; Feng *et al.*, 1991; Kuo and Chiang, 1993; Iwasaki *et al.*, 1994). Most recently, Modl *et al.* (1996) reported the birth of two litters of pigs after transfer of conventionally frozen hatched blastocysts.

Although the aforementioned studies demonstrated that embryos could survive cryopreservation, they contained very low numbers of embryos and subsequent embryo transfers, making it difficult to make firm conclusions. This is not surprising, considering the difficulty and expense in producing large numbers of pig embryos for research use and surgical embryo transfer. Most of the reported studies do not share cryopreservation protocols and many have not been repeated. However, an interesting trend emerged from these reports. It was apparent that survival of pig embryos after cryopreservation may be correlated with stage of embryo development, which may be optimized *in vitro* and peaks during peri-hatching blastocyst progression. They hypothesized the role of embryonic lipid phase changes between the morula/early blastocyst and

the peri-hatching blastocyst stages and possible effects on embryo survival after cryopreservation. Nagashima *et al.* (1992) made substantial contributions to support these observations. They observed that 150–300 μm blastocysts hatched *in vivo* and *in vitro* developed *in vitro* more frequently than did embryos ≥ 300 μm after cryopreservation. Embryo survival after freezing and thawing was also affected by a period of culture before cryopreservation, the presence of protein in the freezing solution and possible breed differences among embryo donors. This effect of developmental stage on embryonic cryosurvival is generally accepted and has been confirmed in subsequent reports (Cameron *et al.*, 1992; Dobrinsky and Johnson, 1994). In short, while intact morulae and early blastocysts do not survive cryopreservation and harsh osmotic disruptions, peri-hatching blastocyst stage pig embryos possess characteristics amenable to cryopreservation.

Vitrification

Vitrification is the rapid cooling of liquid medium in the absence of ice crystal formation. The solution forms an amorphous glass as a result of rapid cooling by direct submersion of the embryo in a plastic straw into LN_2. The glass retains the normal molecular and ionic distributions of a liquid but remains in an extremely viscous, supercooled form (Rall, 1987). The glass is devoid of all ice crystals, and embryos are not subjected to the physical damage that is associated with ice crystal formation (Rall and Fahy, 1985). Vitrification is simple, inexpensive, and has been developed as a viable alternative to conventional freezing of domestic and laboratory animal embryos. Cattle embryos have been successfully cryopreserved with vitrification (Massip *et al.*, 1986; Dobrinsky *et al.*, 1991). More recently, van Wagtendonk-de Leeuw *et al.* (1994, personal communication) demonstrated that vitrification of bovine embryos can be successfully applied in large numbers under field conditions without a significant reduction in pregnancy rate. Overall pregnancy rates after non-surgical embryo transfer were 44.5% (n = 393) for vitrified embryos and 45.1% (n = 335) for conventionally frozen controls.

Until recently, *Drosophila melanogaster* embryos had not been successfully cryopreserved. Like pig embryos, they contain large amounts of lipid and are extremely sensitive to subzero temperatures when supercooled in the absence of ice (Mazur *et al.*, 1992). Unfortunately, embryos died rapidly if they were cooled slowly enough to prevent intracellular ice formation. Mazur *et al.* (1992) suggested that slow cooling injury in *Drosophila* embryos could result from the loss of synchrony of coupled reactions involved in early embryonic development. They speculated that vitrification reduced the possibility of both intracellular and extracellular ice formation, while rapid cooling of embryos could bypass or 'out-race' detrimental chilling-induced cellular changes that take place during slow cooling. Steponkus *et al.* (1990) used vitrification and reported the first successful cryopreservation of *Drosophila* embryos.

Studies in our laboratory have involved development of vitrification procedures for cryopreserving pig embryos. Some vitrification solutions have been shown to be extremely toxic to pig embryos (Weber *et al.*, 1992; Dobrinsky and Johnson, 1994) possibly limiting their use. Dobrinsky and Johnson (1994) applied established vitrification protocols for rodent or bovine embryos using vitrification media based on glycerol-propylene glycol (Dobrinsky *et al.*, 1992), glycerol (VS3a; Rall, 1992; van Wagtendonk-de Leeuw *et al.*, 1994) or ethylene glycol (Kobayashi *et al.*, 1995a,b) and tested their relative toxicity on non-cryopreserved pig embryos produced *in vivo*. Morulae–early blastocysts survived equally across all treatments, while expanded blastocysts developed best after exposure to ethylene glycol and glycerol based media. Hatched blastocysts developed well after exposure to glycerol-based VS3a only, as exposure to ethylene glycol and glycerol-propylene glycol based solutions was detrimental to further development *in vitro*. Therefore, glycerol/VS3a was used as it was least toxic to embryos regardless of embryonic age. After vitrification in VS3a, expanded (27%, n = 64) and hatched blastocysts (39%, n = 84) modestly survived vitrification, while morulae–early blastocysts did not (0%, n = 59). In a subsequent study, Dobrinsky (1993) showed that a culture period before vitrification had no effect on embryo survival following cryopreservation, which is consistent with earlier reports with conventional freezing of embryos (Nagashima *et al.*, 1989a–c; 1992).

Yoshino *et al.* (1993) tested many different vitrification solutions and their cryophysical properties. After cryopreservation, they reported little or no survival *in vitro* and concluded that their vitrification media were extremely toxic to pig embryos. Weber and Youngs (1994) investigated toxicity of cryoprotectant solutions and typical concentrations of sucrose used in the removal of intracellular permeating cryoprotectants on pooled populations of morula to hatched blastocyst pig embryos. They found that sucrose concentrations over 1.0 m l^{-1} were detrimental to further embryo development *in vitro*. Pig embryos were incubated for 30 s in cryoprotectant solutions containing 10, 20, 30, 40 or 50% glycerol, propylene glycol or ethylene glycol, and then diluted in 1.0 mol sucrose l^{-1}. As the concentration of an individual cryoprotectant increased beyond 30%, embryo development decreased. Embryos exposed to glycerol or propylene glycol exhibited poorer development than did embryos placed into ethylene glycol, especially at concentrations of 40% or higher. It should be noted that in these experiments, embryos were placed directly into high concentrations of cryoprotectants without any prior equilibration.

Kobayashi *et al.* (1995a,b) successfully vitrified pig embryos in ethylene glycol and polyvinylpyrrolidone. After vitrification, the respective survivals at 48 h in culture of embryos vitrified as blastocysts, expanded blastocysts and hatched blastocysts were 26%, 33% and 11%. They concluded that extreme chilling sensitivity can be partially circumvented by cooling the embryos rapidly at advanced blastocyst stages. In a subsequent study, Kobayashi *et al.* (1995) showed improved embryo development when using galactose as a cryoprotectant diluting agent. In this study, the respective survivals at 48 h in culture of embryos vitrified as blastocysts, expanded blastocysts and hatched blastocysts were 35%, 96% and 82%. Since their initial reports of these findings in abstract form, no further report in the literature was found on further development of these vitrification procedures and subsequent embryo development *in vitro* or *in vivo*.

In general, all of the reported vitrification studies investigated *in vitro* development after cryopreservation. As of the preparation of this manuscript, there has been no report of live offspring after conventional vitrification of pig embryos. However, it is interesting to note that the trends in embryonic survival after conventional embryo freezing seem to be evident after vitrification and that development to advanced blastocyst stages is paramount to pig embryo survival after cryopreservation.

Cellular Disruption During Cryopreservation

Cryopreservation can be extremely disruptive to the cellular organization of embryos. Ice crystal formation can lyse plasma membranes, storage in liquid nitrogen can denature critical intracellular functions and organelles, and the central cytoarchitecture of a cell can be destroyed. Documenting cellular damage during or after cryopreservation would provide useful, non-empirical information for understanding cellular sensitivities to cryopreservation and would lead to improved protocols for embryo cryopreservation and a better understanding of domestic animal embryology (Dobrinsky, 1996). Recently membrane lipids, intracellular lipids and the embryonic cytoskeleton have been implicated in the hypothermic sensitivity of pig embryos. In the following section more invasive attempts to analyse these cellular disruptions that occur during preservation and subsequent attempts to avoid such damage will be discussed.

Membrane lipids

Polge (1977) first suggested that chilling-induced damage to pig embryos was associated with lipid phase changes within the plasma membranes of blastomeres. There is evidence of phase changes in plasma membrane lipids as temperature is altered (Edidin and Petit, 1977), and the changes are usually reversible. However, during cryopreservation, proteins may interact during cooling that may render phase changes irreversible. This disruption in membrane lipids was identified as a reason why pig embryos may not withstand cooling or cryopreservation (Polge and

Willadsen, 1978; Wilmut 1986). Many attempts have been made to avert damage to plasma membranes during cooling. As was previously discussed, boar spermatozoa are extremely sensitive to cooling and cryopreservation. The addition of phospholipids to boar semen was found to be beneficial at temperatures below 15°C, but was found ineffective with pig embryos (Butler and Roberts, 1975; Niemann, 1985). The addition of fetal bovine serum (Nagashima *et al.*, 1988a,b) to embryo storage media helped improve the sustenance and survival of chilled, expanded and hatched pig blastocysts. Rubinsky *et al.* (1992) proposed that antifreeze glycoproteins isolated from antarctic fishes preserve the structural integrity of the plasma membrane when added to vitrification media. Their use in pig embryo preservation has been limited. Trehalose, a disaccharide known to stabilize plasma membranes and decrease osmotic shock during removal of cryoprotectants (Valdez *et al.*, 1991), was combined with glycerol for conventional freezing of pig embryos by Cameron *et al.* (1992), who reported viabilities of up to 50% after 24 h of culture following cryopreservation. In all, plasma membrane stability and intracellular lipids in general are linked to hypothermic sensitivity of pig embryos.

Intracellular lipids

Pig embryos contain a large amount of sudanophilic lipid in vesicles that is abundant early in development to the blastocyst stage (Norberg, 1973; Niimura and Ishida, 1980; Nagashima *et al.*, 1994). As development continues to the hatching blastocyst stage, lipid content begins to decline (Niimura and Ishida, 1980). The lipid content continues to decline after hatching, coincident with the stage at which pig embryos are able to be cryopreserved (Nagashima *et al.*, 1989 a–c; 1992; 1994; Dobrinsky and Johnson, 1994). It has been suggested that lipid turnover may be important for hatching and normal development, and could be instrumental in increasing the tolerance of pig embryos to cryopreservation. Cooling intolerance was speculated to be due to the high lipid content found in early developing pig embryos (Niemann, 1985; Toner *et al.*, 1986). While it is evident that pig embryos can withstand the osmotic assault of cryoprotectant permeation and withdrawal, cryoprotectants do not protect embryonic lipids or their integrity.

One way to determine whether intracellular lipid is in any way associated with cooling or cryosensitivity of pig embryos is to remove it from the embryo, or delipation, and then cool or cryopreserve the embryo (Nagashima *et al.*, 1994; 1995). When this was done, over 60% of the delipated eight-cell embryos cleaved after chilling to 4°C, whereas all of the controls lysed within 24 h. Fully delipated embryos survived better than partially delipated embryos, while almost 40% developed to the blastocyst stage. It is clear that pig embryos gain some form of tolerance to chilling when their lipid content is reduced. Nagashima *et al.* (1994) proposed that delipation results in changes in the lipid composition of the plasma membranes, and this in turn may limit the extent of phase separation and resultant damage during cooling discussed above.

Lipid vesicles themselves may have a direct effect on embryo survival during cooling (Nagashima *et al.*, 1994; 1995). When pig embryos were held at 15°C, structural changes to the lipid vesicles were observed where they actually coalesce to form larger vesicles (Edidin and Petit, 1977). Lipid vesicles within the cytoplasm have a close spatial arrangement with the smooth endoplasmic reticulum within the embryo (Hyttel and Niemann, 1990) and are thought to play a role of providing nutrition to the cell as well as modifying the physical properties and functions of the cellular plasma membranes (Stubbs and Smith, 1984). During cryopreservation, as the vesicles are at least partially surrounded by endoplasmic reticulum, the resultant loss of cytoplasmic organization may result in irreversible damage to the embryo (Mohr and Trounson, 1981). Removal of the cytoplasmic lipid could therefore eliminate potential cytoplasmic elements causing disruption of the subcellular localization of organelles (Nagashima *et al.*, 1994) such as mitochondria. Although Nagashima *et al.* (1994) have proposed what might be occurring after delipation in relation to cooling sensitivity, no one has explained how pig embryos, with crucial cytoplasmic components removed via delipation, actually compensate for their cytoplasmic losses and maintain development after cooling.

In 1995, Nagashima *et al.* applied delipation principles to conventional freezing of two- to eight-cell pig embryos. More than half of delipated embryos survived cryopreservation, whether they had

been frozen immediately after delipation or after further culture (to determine mitotic competence) prior to freezing, while none of the controls survived. Normal piglets were obtained from at least one recipient after unfrozen delipated and frozen delipated embryos were transferred. This study showed that early cleavage stage embryos survive cryopreservation following delipation, and that somehow, the loss of cytoplasmic lipid is compensated for later in development. These observations of Nagashima and coworkers are landmark observations in understanding the sensitivity of the pig embryo to cooling and cryopreservation, and provide opportunities for studying cryopreservation of oocytes and early stage embryos in other animals that may contain large amounts of cytoplasmic lipid.

Embryonic cytoskeleton

The cytoskeleton is a complex network of protein constituents, actin (microfilaments) and tubulin (microtubules), distributed throughout the cytoplasm of a cell (for review, see Albertini *et al.*, 1987; Dobrinsky, 1996). They give three-dimensionality and mechanical strength to the surface of a cell and provide a system of fibres that impart polarity while regulating cell shape, cell movement, and the plane of cell division. Actin and tubulin filaments bind a variety of accessory proteins that enable them to participate in distinct functions in different regions of the cell, including the plasma membrane. Thus, this highly organized network of filaments forms an internal framework, often referred to as the cytoarchitecture, for the large volume of cytoplasm within the cell.

While the cytoskeleton is important in maintaining structural integrity within the cell, cryoprotectants and cryopreservation disrupt the entire embryonic cytoskeleton. During cryopreservation, cryoprotectants (CPA) are organic solutes used to protect intracellular organelles during long-term preservation in liquid nitrogen. Permeating cryoprotectants, such as glycerol and propylene glycol, among other properties, act to depolymerize microfilaments and microtubules. Depolymerization is beneficial in protecting these cytoskeletal components during osmotic stresses induced by exposure to or removal of cryoprotectants. When coupled with vitrification, however, disruption of the microfilaments or microtubules can be irreversible and lethal to embryos. Total disruption of the plasma membranes of individual blastomeres, as well as lack of blastocoel cavity reformation indicate that many pig embryos cannot tolerate cryopreservation or cryoprotectant treatments and that membrane integrity and metabolic activity are jeopardized.

Some vitrification solutions have been shown to be extremely toxic to pig embryos (Weber *et al.*, 1992; Dobrinsky and Johnson, 1994), and cytoskeletal components react quite differently to CPA exposure (Dobrinsky and Johnson, 1994). Under the influence of high molar concentrations of glycerol needed for vitrification, microtubules in morula to hatched blastocyst stage pig embryos retain polymerization throughout vitrification protocol. Microfilaments are variable in their response to CPA, and the variability is directly related to embryonic stage of development. Prior to cryopreservation of morulae and early blastocyst stage embryos, microfilaments were disrupted by interaction with the CPA. After thawing and subsequent rehydration, cell lysis, membrane disintegration and nuclear damage became evident, microfilaments failed to repolymerize in any organized manner while many nuclei remained crenated and microtubule fluorescence intensity was reduced. Microfilaments of control morulae and early blastocysts equilibrated in vitrification solution repolymerize normally following rehydration while microtubules retain normal staining intensity and nuclei regain normal fluorescent, non-crenated morphology. The irreversible damage of morulae/early blastocyst stage pig embryos is linked to cooling to –196°C but not to the exposure to the CPA used for vitrification. In contrast, some vitrified pig hatched blastocysts retained polymerized microfilaments and microtubules but exhibited regions of intact cortical microfilaments and heterogeneous, cytoplasmic microtubule staining after rehydration. Other vitrified embryos revealed extensive cell lysis and perturbations of normal microfilament and microtubule localization after rehydration. Non-cryopreserved control embryos equilibrated in vitrification solution exhibited normal microfilament repolymerization while microtubules retained normal perinuclear staining patterns.

Table 1. Development of morphologically different stages of swine embryos after cryopreservation by vitrification under the influence of microfilament stabilizer cytochalasin-b

	mVS3a Control			mVS3a + cytochalasin-b		
Stage of development	*n*	dev	% dev	*n*	dev	% dev
MB	17	0	0[a]	17	1	6[a]
XB	27	6	22[ab]	25	15	60[b,z]
HB1 (< 400 μm diameter)	36	10	28[b]	48	43	90[c,z]
HB2 (> 400 μm diameter)	14	4	29[ab]	22	9	41[b]

Data from Dobrinsky *et al.* (1997).
MB: morulae/blastocysts; XB: expanded blastocysts; HB1: hatched blastocysts < 400 μm in diameter; HB2: hatched blastocysts > 400 μm in diameter.
[a–c]Values with different superscripts within individual columns are significantly different ($P < 0.05$); ANOVA–GLM.
[z]Value with superscript within a row is significantly different ($P < 0.01$); chi-square analysis.

The differential survival of pig embryos after vitrification seems to be dependent upon developmental stage of the embryo, which is consistent with numerous reports (see above). The high lipid content found in compacted, pre-blastocyst stage pig embryos may undergo irreversible changes rendering them metabolically useless to the embryo, or lysis of cell plasma membranes may release vital lipid out of the embryo upon rehydration creating a deficiency of lipid for utilization by the embryo. Other membrane-bound structures (such as lysozymes) may release their contents into the cytoplasm or be rendered useless to the embryo. Microtubule damage from cryopreservation might also affect the mobility and transport of lipid and other components in the embryo, thus breaking the supply of substrate needed during blastocyst formation in the embryo. Microfilament damage may be so extensive as to disable any structural support to the plasma membrane (for review, see Dobrinsky, 1996).

An intact cytoskeleton is the highway for cytokinesis and karyokinesis, and if disrupted in any way, the mitotic cell cycle will cease. Maintaining the integrity of the cytoarchitecture within an embryo during cryopreservation is of the utmost importance, especially with pig embryos. Because of the cooling sensitivity, and fragile plasma membranes, finding ways to overcome or prevent cytoskeletal disruption will improve the survival of pig embryos during and after cryopreservation.

Cytoskeletal stabilization

Cytoskeletal stabilizers, such as the cytochalasins, have been used extensively in reproductive biotechnology for studies dealing with micromanipulation of embryonic development (McGrath and Solter, 1983; Surani *et al.*, 1984). It is generally accepted that cytochalasins are a microfilament inhibitor (Figure 1) disrupting actin polymerization by blocking monomer addition at the fast-growing end of the polymers (Theodoropoulos *et al.*, 1994), thus preventing cytokinesis without affecting karyokinesis (Modlinski, 1980; Cuthbertson, 1983). Treatment of cells with cytochalasins makes the plasma membrane less rigid and more elastic so microfilaments are not disrupted during extensive micromanipulation (McGrath and Solter, 1983).

Dobrinsky *et al.* (1997; submitted) have documented microfilament damage during swine embryo vitrification and the use of cytochalasin-b before and during cryopreservation (Fig. 1; Table 1) to help deter damage and stabilize the plasma membrane (Prather and First, 1986; Dobrinsky, 1996; Dobrinsky *et al.*, 1997). As reported in previous studies, morulae or early blastocysts do not survive cryopreservation. Treatment with cytochalasin-b did not improve their viability after vitrification. However, cytochalasin-b treatment provided a threefold improvement in survival of

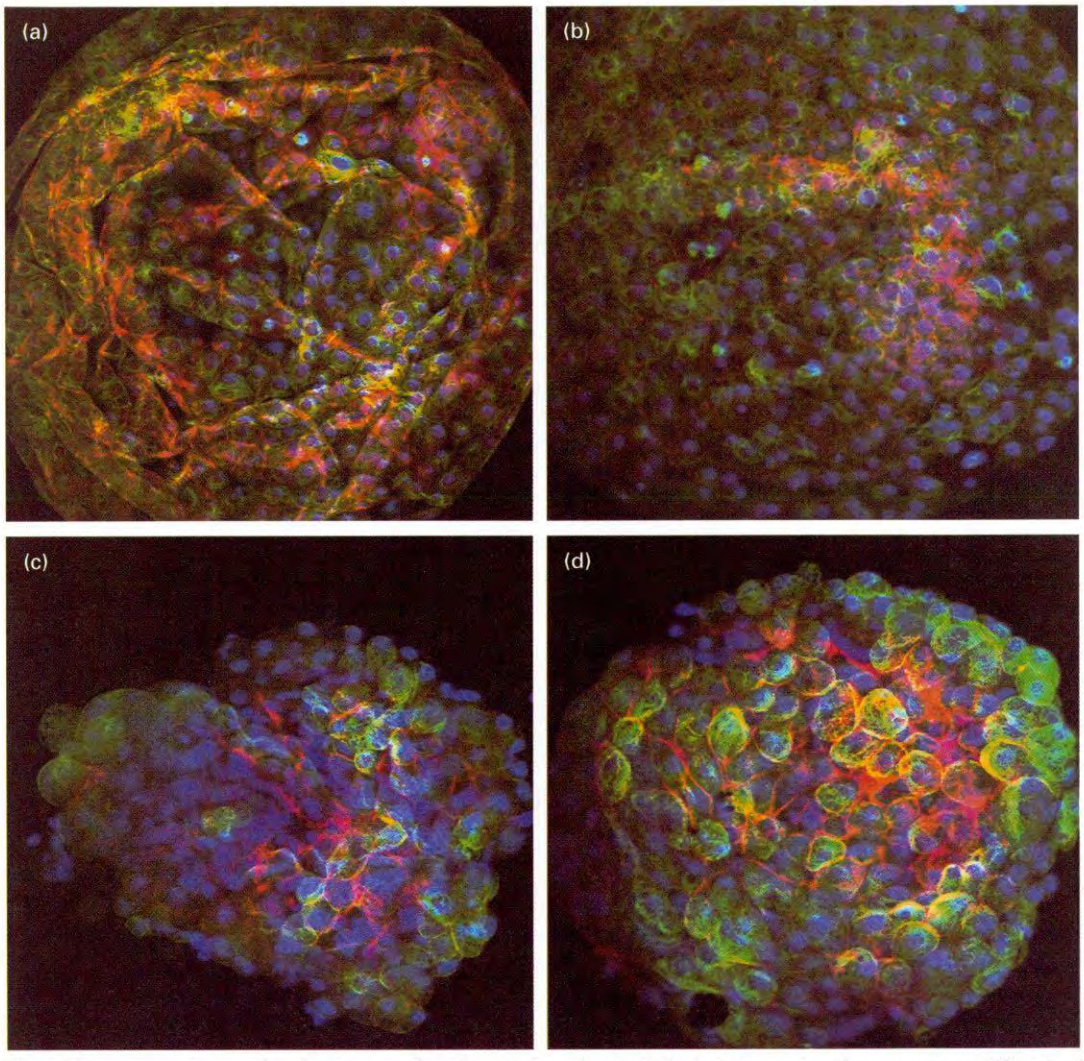

Fig. 1. Laser scanning confocal micrographs of control and cytochalasin-b treated and cryopreserved hatched blastocyst stage pig embryos. In the micrographs, the red label is Texas Red-X phalloidin staining of filamentous actin (microfilaments), the green label is FITC staining of α- and β-tubulin (microtubules), and the blue label is Hoechst 33342 staining of cell nuclei. (a) Non-treated control hatched blastocyst; note the intact actin labelling consistent and prevalent at all cell to cell borders. (b) Cytochalasin-b treated hatched blastocyst: after incubation in cytochalasin, note the reduction of phalloidin staining at the cell to cell borders, indicating depolymerization of microfilaments, while microtubules and the nuclei remain mostly unchanged. (c) Non-treated, vitrified, warmed hatched blastocyst, cultured for 2 h; note the disrupted spherical orientation of the embryo proper, and inconsistency of cellular organization. Part of the embryo remains intact, while the remainder of the embryo has lost all normal cellular organization and staining patterns of the cytoskeleton. (d) Cytochalasin-b treated, vitrified, warmed hatched blastocyst, cultured for 2 h; note the reformation of normal staining patterns of the cytoskeleton, indicating normal repolymerization of microfilaments and microtubules, as well as the reformation of a normal spherical dimension for the embryo.

expanded (60%) and hatched blastocysts less than 400 μm in diameter (90%). Although cytochalasin-b-treated expanded blastocysts had improved viability after vitrification, their development in terms of blastocoel reformation and proliferation in culture following vitrification was still lower than that

of hatched blastocysts. These data are the highest rates of *in vitro* development after cryopreservation ever attained in our laboratory, and results are consistent across embryo donors. Cellular analysis with laser scanning confocal microscopy revealed reduced fluorescence intensity of microfilaments in cytochalasin-b treated embryos (Fig. 1). Non-cytochalasin treated vitrified embryos exhibited partial or major cytoskeletal disruptions within 2 h after rehydration, whereas cytochalasin treated vitrified embryos mostly exhibited normal repolymerization of microfilaments and other cytoskeletal components after vitrification. These experiments show that the cytoskeleton is affected during vitrification, and that microfilament depolymerization prior to cryopreservation significantly improves expanded and hatched blastocysts development.

Most recently, we (Dobrinsky, V. Pursel and L. Johnson, unpublished observations) have established four pregnancies after transfer of cytochalasin-b treated vitrified hatched blastocysts into seven recipient gilts. Hatched blastocysts < 400 μm were treated with cytochalasin-b, vitrified and then stored in LN_2 for at least one week. Natural cyclic gilts, with normal oestrous cycles and sound confirmation were selected as recipients and used on day 6 (day 0 = oestrus) of the oestrous cycle. Straws containing embryos were warmed and embryos were diluted from cryoprotectants, rehydrated, cultured in BECM-3+10% FBS for up to 6 h, and then transferred via transoviductal catheterization into a single uterine horn.

Conclusion

Methods for preservation of pig embryos are in early stages of development, as are other technologies in pigs, such as non-surgical embryo transfer (Li *et al.*, 1996), that could make embryo cryopreservation practical and usable by the producer. Technologies available for embryo transfer in cattle, such as non-surgical embryo recovery and *in vitro* embryo production, are not readily available to the pig industry or are in early stages of development, making it impractical for the producer to use these biotechnical advances in swine. Much of the early research in preservation of pig embryos was observational. A few reports mentioned inconsistent rates of embryo survival both *in vitro* and *in vivo*. However, since the late 1980s, techniques for preservation of pig embryos have advanced. The pig embryo is known to be extremely sensitive to hypothermic conditions. Its intracellular lipids play a major role in this sensitivity. Delipation and conventional freezing or vitrification are viable methods for embryo preservation, but the test of these methods is a repeatable production of live pigs. Embryo delipation is somewhat impractical as it relies on invasive manipulation of the embryos before preservation, only to inflate the cost of its application. Vitrification is a simple alternative for cryopreserving pig embryos, but its efficacy is yet to be realised in providing high rates of live offspring after transfer. Other advances in pig reproduction and biotechnology, such as *in vitro* embryo production and non-surgical embryo transfer, will only enhance the use of embryo cryopreservation. Alternative methods are needed to preserve valuable genetic resources in a reasonable and economical manner. More invasive research needs to be conducted to characterize cellular and molecular disruption during and after cryopreservation, so that better methods for preserving pig embryos can be developed.

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