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REVIEW

Cryopreservation of farm animal gametes and embryos: recent updates and progress

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Abstract Cryopreservation has undergone tremendous advances and is widely used in animal production based on decades of study of cellular permeability, freezability and empirical generalization. Several improvement are particularly important: the cryopreservation protocol has been continuously refined over the years to achieve greater reproductive performance; cryoprotective agents are more effective and less toxic than previously; there has been significant innovation in advanced cryopreservation systems and carriers. Despite this, there are still problems that urgently require practical solutions, such as remedies for cryodamage and encouraging the use of frozen–thawed porcine sperm in pig production.

Keywords vitrification, gametes, embryo, animal production, cryoprotective agent, freezability

1 Introduction

Because of its important contribution to animal production, genetic resource preservation, embryo biotechnology and human assisted reproduction technology, there has been much progress in cryopreservation over a long period, which has been driven predominantly by research in humans, cows, sheep, pigs and mice[1]. Cryopreservation protocols have evolved substantially for gametes, embryos and reproductive tissues (ovarian and testicular tissue), resulting in a significant increase in the proportion of fertilizable sperm, viable oocytes and transferable embryos.

The cattle industry has benefited the most from the application of cryopreserved semen or embryos over past decades. International exchange of high quality breeder bull resources has accelerated with semen cryopreservation. Cryopreserved semen allows the mating of female cattle that is neither bound by time nor place, while minimizing the risk of disease transmission[2]. The USDA indicated that frozen bovine sperm and artificial insemination contributed 97% to the genetic improvement of cow herds in 2008. Cryopreservation enabled embryo transport to be more practical and cost effective; remarkably, 30 years of international trade with frozen embryos has not resulted in the transmission of a single infectious disease agent[3]. According to the census from the International Embryo Technology Society, in 2015, more than 60% of bovine embryo transfer was conducted using frozen–thawed embryos[4].

There are two strategies that may fulfill the requirements for successful cryopreservation of mammalian gametes and embryos: slow freezing (programed) and vitrification. A typical cooling rate of slow freezing is about 1 °C·min^{-1} which is appropriate for many mammalian cells after treatment with cryoprotective agents (CPAs) such as glycerol or dimethyl sulphoxide (DMSO), and this rate can be achieved by using devices such as a rate-controlled freezer or a benchtop portable freezing container[5]. Vitrification is an ultrarapid cooling technique, for which the protocols are simple, allowing cells and tissue to be placed directly into CPAs and then plunged directly into liquid nitrogen. In vitrification, ice crystal formation is prevented by using high concentrations of CPAs and high cooling and warming rates[6]. Although vitrification as a method of cryopreserving embryos was developed in the mid-1980s as an alternative to the then standard slow freezing, its suggested advantages (simplicity, cost and speed) have had little impact on commercial embryo transfer operations and its application has remained largely confined to research studies[7]. In comparison to slow-freezing, which requires more than 2h, vitrification only requires a few minutes, minimizing the time of exposure to subphysiological conditions[8]. Vitrification also has the attraction of avoiding the need for expensive equipment required for cryopreservation by

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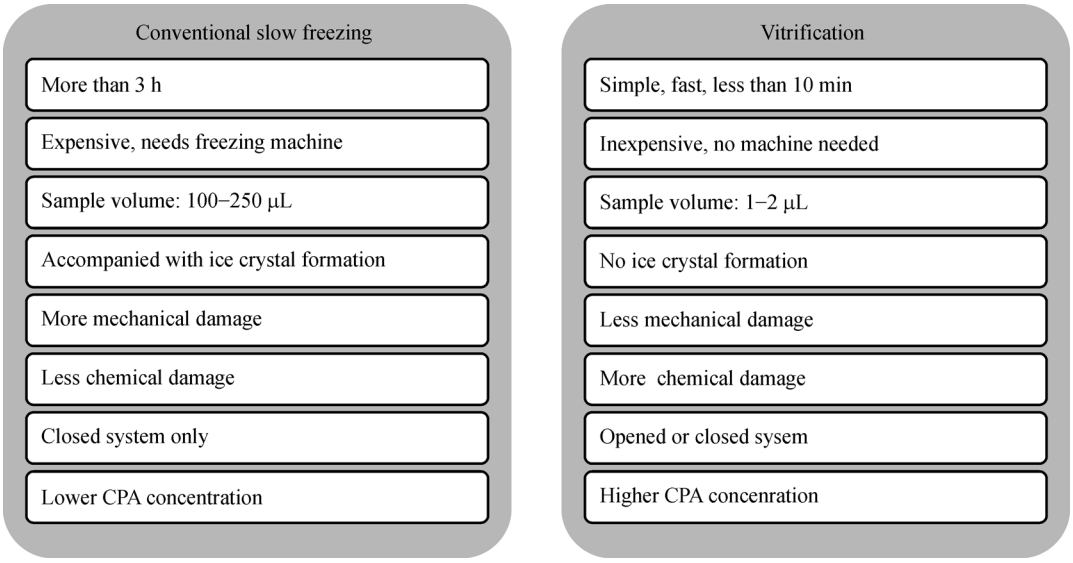

Fig. 1 Comparison between slow freezing and vitrification[9]

slow freezing (Fig. 1). It is notable that the literature on cryopreservation technology makes a distinction between '*thawing*' as applied to embryos and oocytes preserved by slow freezing and '*warming*', which is the term used in bringing embryos back to ambient temperature after vitrification.

In this review, the major updates and progress in the development of cryopreservation technologies are summarized in order to highlight aspects that still require improvement and to encourage further developments in this field.

2 A short overview of the basic principles of cryopreservation

2.1 The physical chemistry of cryopreservation and warming

Although the cytosol of gametes or embryos contains a high proportion of water[10], it is still feasible for them to be vitrified. Water is not very viscous and it can be vitrified only by an extremely rapid flash-freezing of a small sample[11], about 3×10^6 °C·s^{-1} from room temperature to −135°C[12], consequently, dehydration of the sample is critical, and is achieved by exposure to high concentrations ($\geqslant 6$ mol·L^{-1}) of CPA as pretreatment, followed by plunging the sample into liquid nitrogen. Under such a rapid cooling rate, water molecules do not have time to arrange themselves into a crystalline lattice structure[13] and the physiological structure of gamete or embryo can be maintained[14]. Using the standard French mini-straw as an embryo container, vitrification enabled a maximum cooling rate of about 2×10^3 °C·min^{-1}, while Vajta's OPS method permits much higher cooling and warming rates ($> 2 \times 10^4$ °C·min^{-1}).

Shrinkage occurs during vitrification (Fig. 2), so during warming the gamete or embryo is placed into solution at lower concentration and the CPAs are replaced with water and gradually swell to its original size. Thawing solution contains sucrose, which does not penetrate through the cell membrane because of its size, but it does control the rate of swelling[16], striking a balance between swelling and shrinkage.

2.2 Cryoprotective agents

Some amphibians have freezing resistance due to glycerol manufactured by their livers[17]. Glycerol is an antifreeze, like ethylene glycol (EG) used as an automobile antifreeze, and reduces ice formation and lowers the freezing point, which can make frozen water look like glass — with no crystal formation — a process called vitrification. In 1959, DMSO was demonstrated to be useful as a CPA owing to its high penetrating rate[18], though it can be more toxic at higher temperatures[19]. In 1972, eight cell mouse embryos were cryopreserved to liquid nitrogen temperature and rewarmed to obtain live mice, by slow cooling and skillful combination of DMSO with glycerol[20]. The higher the concentration of CPA, the higher the glass transition temperature, thus lowering the chance of ice nucleation and crystallization[21].

Some non-permeating CPAs like trehalose, sucrose and ficoll, are also added because they can increase the osmotic pressure which is conducive to both dehydration and penetration of EG and DMSO. It was also demonstrated that trehalose could improve the freezing tolerance of oocytes[22–24]. Then during thawing, an appropriate

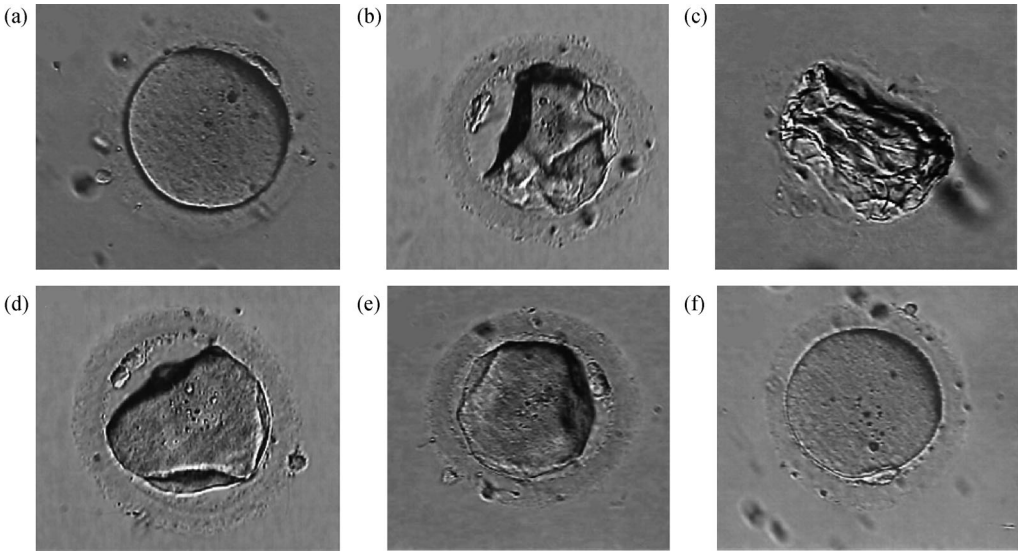

Fig. 2 The morphological changes of human MII oocytes in vitrification solution[15]. (a) Before vitrification; (b) in equilibration solution; (c) in vitrification solution; (d) in dilution solution; (e) in washing solution; (f) in the culture media 5 min after final washing.

concentration of sucrose can be used to remove the permeated CPAs by establishing a proper osmotic pressure and this process is called detoxification.

2.3 Volume

Smaller volumes allow more efficient heat transfer, thus facilitating rapid cooling rates. Furthermore, the smaller the volume, the higher the probability of vitrification[25]. Compared to freezing in 0.25-mL straws, the cooling rate is enhanced two to six times when freezing occurs in a more refined device, such as open-pulled straw or electron microscope grid[26]. Decreasing the vitrified volume and increasing the cooling rate allow a moderate decrease in CPA concentration so as to minimize its toxic and hazardous osmotic effects[27]. It was shown for oocytes and embryos that increasing the cooling rate improves survival rates by up to 37%[1].

3 Cryopreservation of mammalian preimplantation embryos

Embryo cryopreservation has been widely used in animal reproduction since a calf was born from frozen–thawed embryos for the first time in 1973[28,29]. Vitrification of embryos was invented in 1985[30] and successive breakthroughs have been achieved for farm animals including cattle[31], goats[32], sheep[33] and pigs[34] (Table 1).

There are several important characteristics for embryo cryopreservation, for example, the permeability of the plasma membrane of embryos varies during developmental stages, because permeation velocity improves along with the formation of blastomeres[35]. Different farm animals have distinct responses to freezing, i.e., freezability, for example, porcine embryos are particularly sensitive to low temperature due to their high lipid content[36,37], and the freezability of ovine embryos increases along with their development[38]. Studies indicate an increased survival rate, development potential and freezability with development after vitrification of ovine four cell embryos, eight cell embryos, 16-cell embryos, morulae and blastocysts[39,40].

Table 1 Milestones in vitrification of embryos[3]

Year	Species	Researcher
1985	Mouse	Rall and Fahy
1986	Cow	Massip et al.
1986	Hamster	Critser et al.
1988	Rat	Kono et al.
1989	Rabbit	Smorag et al.
1990	Sheep/goat	Scieve et al.
1994	Horse	Hochi et al.
1998	Pig	Kobayashi et al.

As the most widely used embryo biotechnology, cryopreservation of bovine embryos has developed rapidly and according to the data from the International Embryo Technology Society, more than 300000 frozen–thawed embryo transfers were conducted around the world in 2015[4]. Studies indicated that use of conjugated linoleic acid[41] or lipolysis agents[42] during bovine embryo culture and cryopreservation could enhance the post-warming survival rate. Embryo culture in medium with

lower concentrations of serum and metabolism regulator, which could inhibit lipogenesis, also led to a higher post-warming survival rate[43]. Similar outcomes have been obtained by adding cytochalasin or using centrifugation to decrease lipid content[44], and addition of caspase inhibitor Z-VAD-FMK could improve freezability of *in vitro* derived bovine embryos[45]. To improve embryo thawing, the one-step method for direct nonsurgical transfer of frozen–thawed bovine embryo[16] has proven to be efficient for the cattle industry because it simplifies the thawing procedures and prevents embryo loss during thawing.

Vitrification of ovine embryos has become common practice in animal production for nearly 30 years since the first success in 1990. Pregnancy rate and lambing percentage were significantly higher in vitrified-warmed blastocyst transfer using open-pulled straw (OPS) vitrification compared to slow freezing[46]. It was demonstrated that DMSO could lead to a lower development rate after ovine embryo vitrification compared to EG[47]. To improve culture systems, a growing number of studies have shown that adding an antioxidant such as melatonin[48,49], or lipid lowering agent, such as conjugated linoleic acid[50], can improve the outcome after vitrification. Adding cathepsin B to *in vitro* cultures cannot only improve quality and quantity of ovine blastocysts but also improve the cryo-survival of *in vitro* derived blastocysts[51]. Also, vitamin K2 can improve the developmental competency and freezability of *in vitro* derived ovine blastocyst[52].

Progress in porcine embryo vitrification has been slow compared to other farm animals and there was no successful frozen-warmed porcine embryo transfer until 1989, probably because of its high intracellular lipid content[53]. Neither mechanical methods like centrifugation[54,55] nor adding chemicals into the culture medium[56] to lower the lipid content within blastocysts was able to improve the freezability or the post-warming survival rate. The application of hydrostatic pressure before vitrification has improved blastocyst survival rates after warming, to over 10%[57]. Disrupting the lipid bilayers by micromanipulation and then centrifuging embryos before vitrification has improved post-warming survival, and using this approach, vitrification in a closed system was as successful as using open-pulled straws, which was a major step forward in porcine embryo cryopreservation[58]. Carboxylated ε-poly-L-lysine is an effective CPA for porcine embryo vitrification and it can improve the developmental ability of pig embryos vitrified at the pronuclear stage[59]. Vitrification of expanding blastocysts using cryotop has given a higher survival rate and a piglet was successfully born after blastocyst transfer[60].

4 Cryopreservation of mammalian oocytes

Recent researches have focused on refinement of oocyte vitrification, specifically, screening optimal CPAs, selecting cryopreservation carriers, and refining the timing of pretreatment and vitrification procedures. Apart from empirical generalization, cryopreservation protocols need to be specialized based on the biological characteristics of oocytes of different species.

One particular challenge pertaining to mammalian oocyte cryopreservation is their extremely high cellular volume compared with other cell types, making them particularly sensitive and even more susceptible to intracellular ice formation during the process of cryopreservation due to a lower surface-to-volume ratio[61]. Also, the elasticity of oocyte membrane is inferior to that of the embryo, which could explain why it is easily injured during freezing[62]. Moreover, CPAs can induce an increase of cytosolic calcium concentration in oocytes during vitrification and warming. For instance, DMSO stimulates the release of cytosolic calcium and EG improves calcium influx; the increase of cytosolic calcium concentration induces zona hardening and affects the penetration of sperm and fertilization[63]. Cytosolic lipids are critical to oocyte maturation and development, but are the biggest obstacle to cryopreservation by increasing freezing sensitivity[64], especially in porcine oocytes, which contain 6.8 times as much lipid as mouse oocytes[65]. From the perspective of developmental stage, under the same treatment, MII oocytes have a higher post-warming survival rate than germinal vesicle (GV) oocytes but there is no obvious difference in their subsequent development[66]. One study indicated that GV breakdown oocytes have a better development compared to GV or MII oocytes after vitrification[67].

Given the particular biological characteristics of bovine oocytes, they have better freezability than porcine or ovine oocytes[68], consequently, the frozen–thawed bovine oocytes are more likely to develop into blastocysts after *in vitro* fertilization. The first calf from a frozen–thawed oocyte was born in 1992[69] and the first successful vitrification of a bovine oocyte by OPS was in 1998[13]. Vitrified-warmed bovine oocytes producd by the OPS method can be used for somatic cell cloning, and a cloned calf was successfully born after embryo transfer[70]. Improvements of bovine oocyte cryopreservation have been made over the years, for example, using macromolecule polymers as CPAs to lessen toxicity[71], using cryotop can lead to a better outcome of vitrified bovine GV and MII oocytes[72], and using solid-surface vitrification reduces the ultrastructural injuries[73]. Docetaxel treatment before vitrification can significantly decrease injury to the cytoskeleton of bovine oocytes, thereby improving their post-warming survival rate and development potential[74]. Other chemicals, such as conjugated linoleic acid[75], L-carnitine[76], glutathione[77] and a cAMP agonist[78] have also improved outcomes. Cholesterol, coenzyme Q10, BAPTA-AM (Ca^{2+} chelator) and ruthenium red have also improved the freezability of *in vitro* matured bovine

oocytes[79–81]. Liquid helium vitrification of immature bovine oocytes had better outcomes for reducing injury to the cytoskeleton structure and improving the viability compared with liquid nitrogen vitrification[82].

Progress in ovine oocyte research has lagged far behind that carried out with bovine oocytes. Most studies have focused on optimizing the oocytes stage, cryopreservation carriers and specialized drugs. The first successful vitrification (using a cryoloop) of ovine GV oocytes was reported in 2013; vitrified oocytes had the ability to mature, to be fertilized and to subsequently developed *in vitro* to produce good-quality blastocyst embryos at frequencies comparable to those obtained using fresh oocytes[83]. Also GV oocytes vitrified by cryotop had a higher polar body extrusion rate[84]. Open vitrification carriers like cryotop and cryoloop have proven to have better outcomes on ovine MII oocyte vitrification compared to closed or half-closed systems, having higher percentage of survival[85], cleavage[86] and *in vitro* maturation[87], but there was no significant difference between OPS and cryoloop with respect to the rate of blastocyst formation[88]. The addition of angiotensin II to the *in vitro* maturation and *in vitro* culture media could improve blastocysts formation in vitrified sheep oocytes[89]. To maintain the oocyte cytoskeleton of MII oocyte during vitrification, pretreatment with 7.5 $\mu g \cdot L^{-1}$ CB or 0.5 $\mu mol \cdot L^{-1}$ taxol has been shown to improve outcomes[90], but CB did not improve the survival and development of GV oocytes[83,91]. Additionally, 1 min treatment with 2.5 $\mu mol \cdot L^{-1}$ ionomycin effectively improved the activation of vitrified-warmed MII oocytes and led to a higher blastocyst rate[92].

After decades of study, vitrified-warmed porcine GV and MII oocytes can now be developed to healthy embryos after *in vitro* maturation and fertilization, and piglets were successfully born in 2014[93,94]. For improved carriers, there is a consensus that cryotop is superior to OPS[95,96]. Studies on permeating CPAs indicated that EG + DMSO and EG + propylene glycol are both efficient in improving post-warming survival after vitrification[97,98]. For non-permeating CPAs, adding *Lycium barbarum* polysaccharides is beneficial for GV oocyte vitrification[99]. Before vitrification, taxol treatment can maintain spindle integrity, spatial distribution of mitochondria and lipid droplets[100,101]. It also increases the percentage of vitrified-warmed MII oocytes that develop into blastocysts after parthenogenetic activation[102]. It has been demonstrated that the cytosolic lipid content of porcine oocyte can be lowered by adding Forskolin (stimulator of lipolysis) to improve the freezability of *in vitro* maturated porcine oocytes[103]. The addition of antioxidants, such as glutathione, taurine, vitamin E and resveratrol, minimizes oxidative damage and reduces the rate of apoptosis[104–108]. Thioglycol can counter the increase in reactive oxygen species level induced by vitrification[109]. During *in vitro* maturation of vitrified-warmed porcine GV oocytes, adding cyclosporine A and BAPTA-AM to the culture medium can decrease mitochondria calcium concentration, and increase survival and maturation rate[110].

5 Cryopreservation of mammalian semen

Sperm cryopreservation has the longest history and is the most widely used in animal production and human reproductive medicine, due to high freezability, large numbers and straightforward protocols[111]. Successful sperm cryopreservation is based on the peculiar structure of sperm. The head of the sperm contains lipoprotein and enzymes used for penetrating the oocyte, with weaker freezability. The midpiece has a central filamentous core with many mitochondria spiraled around it for ATP production. The tail or flagellum executes the lashing movements[112], and had stronger freezability than the head because of their solid structure and lower water content.

Bovine sperm is not sensitive to low temperature, while porcine and ovine sperm are quite sensitive to temperature changes, and more likely to suffer from cold shock between 5 and 22°C leading to rapid loss of vitality. Compared to bovine sperm, the porcine sperm membrane contains less lecithin[113], which is necessary for maintaining membrane fluidity. Anti-oxidase is easily lost during cryopreservation, combined with high content of unsaturated fatty acid in farm animal sperm, resulting in their vulnerability to oxidant damage[114]. It was reported that the expression of heat shock protein 90 in porcine sperm was significantly downregulated after cryopreservation[115] and decreased to 64%[116] compared to fresh sperm, which might be related to the vitality loss after thawing. In general, freezing-thawing of mammalian sperm harms the cell, the extent of that damage varies across species and depends heavily upon the sperm resilience to withstand cryopreservation procedures[111,117].

Egg yolk-sodium citrate diluent (EYC) was the first extender used for bovine sperm preservation[118], and was gradually replaced with tris-buffered egg yolk (TRIS-EY)[119] or tris-fructose yolk-glycerol[120]. Accordingly, tris and citrate are now used as the major components of bovine sperm extender for cryopreservation and widely used in industry. Bovine sperm cryopreservation was developed in the 20th century, when glycerol was used as a CPA for mammalian sperm[121]. Glycerol has been demonstrated to be the best CPA with an optimal concentration that ranges from 2% to 3%[122] and cryopreserved bovine sperm is often packaged in 0.25-mL or 0.5-mL straws with EYC or TRIS-EY combined with glycerol[123]. For non-permeating CPAs, sucrose has been shown to have better efficacy than trehalose[124]. Low density lipoprotein can be substituted

for egg yolk because it can counter the injuries induced by cold shock, as well as maintaining the physiological structure of sperm, resulting in higher vitality after thawing[125,126]. Other additives such as vitamin E or melatonin, have been found to increase the integrity of acrosome, improve vitality, decrease abnormality rate and prevent oxidative damage[127–129].

The earliest report of ovine sperm cryopreservation was in 1937[130] and later, Smirnov successfully cryopreserved 0.05–0.10 mL of ovine sperm without using glycerol[131,132]. Ram has distinctive seminal plasma containing phospholipase A, which can hydrolyze the lecithin in egg yolk to fatty acid and lysolecithin, then induce the condensation of egg yolk[133] and the incidence of acrosome reaction[134]. It turned out that the development of ovine sperm cryopreservation was not as fast as for bovine sperm. In the 1960s, the conception rate from frozen–thawed ovine sperm was 37.9%–66.2%[135,136], but improvements in cryopreservation have been made over the decades. Trehalose was introduced as an efficient non-permeating CPA to cryopreserve ovine sperm, leading to a higher vitality along with increasing concentration of trehalose, reaching more than 60%[137]. Similarly, sucrose was proved to be capable of effectively preserving sperm morphology and DNA integrity[138]. This was explained by studies which demonstrated that hypertonic extender was more suitable for ovine sperm cryopreservation since it can withstand twice the osmotic pressure of an isotonic glucose solution[139]. Centrifugation was introduced to remove seminal plasma, resulting in higher post-warming survival rates and integrity of acrosome[134,140]. However, the conception rate of frozen–thawed ovine sperm could not be stabilized at more than 60%[141]. Granule frozen and straw frozen sperm are commonly used in sheep production, but there is no study on frozen ovine sperm thawing, and the diluents for thawing are basically 2.9% sodium citrate, inositol-citrate, glucose-citrate or fructose-citrate.

Piglets from frozen–thawed sperm were first born in 1957. The 1970s represented a significant period of advancement for porcine sperm cryopreservation with the establishment of two methods. The Beltsville method[142] used carbonic ice and the Westendorf method[143] used liquid nitrogen vapors, however, cryopreservation success was further increased through the introduction of controlled-rate freezers, which gave better results (i.e., sperm quality at post-warming) than the standard method (i.e., nitrogen vapors in a polystyrene box containing liquid nitrogen)[144,145]. Porcine sperm cryopreservation extenders including the buffered type, such as EY-Glucose[146], EY-Sucrose-EDTA-calcium or magnesium salts[147] and EY-lactose[148], or unbuffered type, such as Beltsville F5 (BF5)[142], EY-glucose-citrate-EDTA-potassium-unitol-urea[149] and tris-glucose-EDTA-EY[150]. Studies on CPAs indicated that using 0.09 $g \cdot mL^{-1}$ low density lipoprotein to substitute EY[151] can lead to better post-warming sperm quality and glycerol combined with acid amides was better than glycerol alone[152]. Also, adding hyaluronan[153], cholesterol[154] or butylated hydroxytoluene[155] to extenders, diluting semen and adjusting the pH to 8 could improve quality maintenance[156]. Cryopreserved porcine sperm are often packaged in 0.5-mL straws and the most recent figures indicate that farrowing rates following artificial insemination with frozen–thawed sperm are around 75%–80%[144].

6 Conclusion and future perspectives

While there has been considerable success with cryopreservation of oocytes, embryos and semen in farm animals, this technology still requires refinement and further studies of the basic principles is needed so that greater success and higher efficiency can be achieved.

Oocyte and embryo cryopreservation are applied across many areas of animal production, and cryopreservation protocols are established according to different objectives because cellular characteristics vary among different species, and even within a species at different developmental stages. For example, for porcine oocyte or embryo cryopreservation, high hydrostatic pressure application may be worthy of further development as a potential way to improve results, in combination with the use of improved vitrification solutions and possibly delipidation of the cytoplasm, it could yield better and more consistent results[57]. The prominence of cryodamage in this species deserves further investigation as a possible limiting factor for successful vitrification. For ovine oocytes, further attention to the effects of vitrification on transcription factors could be fruitful for overcoming the developmental blocks seen in this species. Furthermore, the interaction between cytoplasmic calcium and extracellular fetal calf serum with transcription factor expression warrants further study[86,157,158]. Generally speaking, studies on molecular and biochemical evaluation of CPAs and careful selection of less toxic CPAs, close monitoring of their temperature, time of exposure, concentration, and their stepwise addition and removal from cells[9] are needed.

The use of frozen–thawed porcine sperm is still considered suboptimal[159] because of the specific features of the sperm cryopreservation protocols and pig breeding[160]. Future studies on cryodamage (Fig. 3) should focus on physiological structures (integrity of sperm membrane, sperm chromatin and mitochondrial function), factors that influence ejaculate freezability (season, diet, genetic differences, spermatogenesis and epididymal maturation), and identification of effective additives and freezability markers.

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Compliance with ethics guidelines Zhengyuan Huang, Lei Gao, Yunpeng

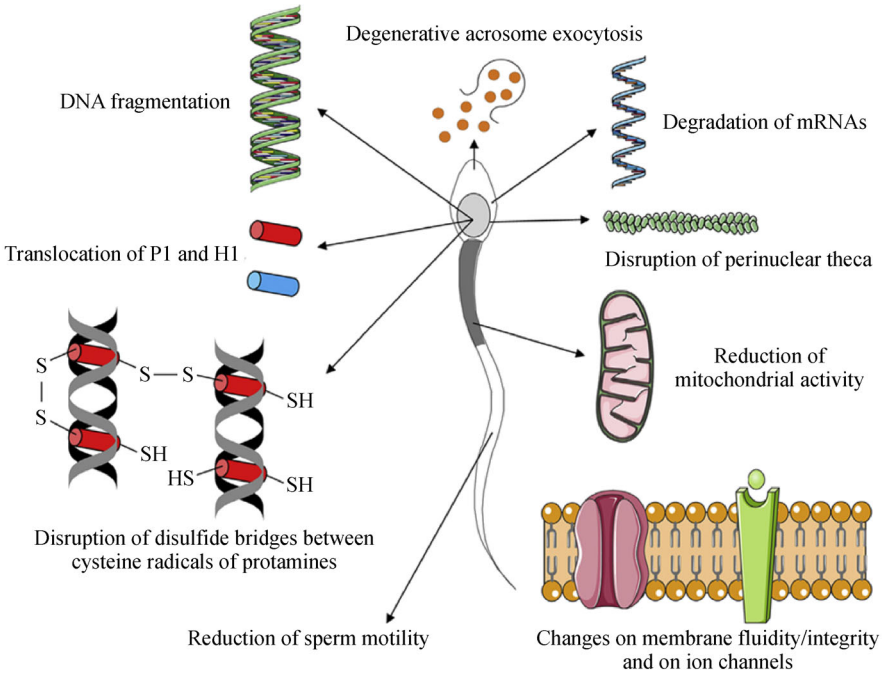

Fig. 3 Main damage inflicted by freezing-thawing procedures on porcine sperm[160]

Hou, Shien Zhu, and Xiangwei Fu declare that they have no conflicts of interest or financial conflicts to disclose.

This article is a review and does not contain any studies with human or animal subjects performed by any of the authors.

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