Cryopreservation of mammalian oocytes and embryos: current problems and future perspectives

MOUSSA Mahmoud1,2,3,4,5,6, SHU Juan1,2,3,4, ZHANG XueHong5 & ZENG FanYi1,2,3,4*

1Shanghai Institute of Medical Genetics, Shanghai Children’s Hospital, Shanghai Jiao Tong University, Shanghai 200040, China; 2Institute of Medical Science, School of Medicine, Shanghai Jiao Tong University, Shanghai 200025, China; 3The Key Lab of Embryo Molecular Biology, Ministry of Health, China, and Shanghai Lab of Embryo and Reproduction Engineering, Shanghai 200040, China; 4Shanghai Tao Tao Transgenic Corp., LTD, Shanghai 201604, China; 5Department of Bioengineering, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China; 6Department of Theriogenology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia 41522, Egypt

Received November 28, 2013; accepted February 3, 2014; published online August 6, 2014

Cryopreservation techniques for mammalian oocytes and embryos have rapidly progressed during the past two decades, emphasizing their importance in various assisted reproductive technologies. Pregnancies and live births resulting from cryopreserved oocytes and embryos of several species including humans have provided proof of principle and led to the adoption of cryopreservation as an integral part of clinical *in vitro* fertilization. Considerable progress has been achieved in the development and application of the cryopreservation of mammalian oocytes and embryos, including preservation of the reproductive potential of patients who may become infertile, establishment of cryopreserved oocyte banks, and transport of oocytes and embryos internationally. However, the success rates are still far lower than those obtained with fresh oocytes and embryos, and there are still obstacles that need to be overcome. In this review, we address the major obstacles in the development of effective cryopreservation techniques. Such knowledge may help to eliminate these hurdles by revealing which aspects need improvement. Furthermore, this information may encourage further research by cryobiologists and increase the practical use of cryopreservation as a major part of assisted reproductive technologies for both humans and animal species.

cryopreservation, mammals, oocytes, embryos, cryoinjuries

In the past few decades, assisted reproductive technology (ART) has been used to compensate for infertility of couples and commercially valuable animals. There have been improvements in many ART techniques, resulting in a significant increase in the proportion of viable oocytes and transferable embryos. However, surplus oocytes and embryos have created the need to develop cryopreservation techniques.

Cryopreservation of female gametes and embryos emerged after investigation of the effect of low temperature storage on rabbit oocytes, zygotes, and embryos by Chang et al. [1,2]. Subsequently, cryopreservation protocols have evolved substantially for gametes, embryos, and ovarian tissues. In 1977, the first live births from cryopreserved ovulated mammalian oocytes were reported in mice [3] followed by a number of species including human [4], rabbit [5], cow [6], and horse [7].

There are two strategies that may fulfill the requirements for successful cryopreservation of mammalian oocytes and embryos: slow freezing and vitrification. Several studies
have indicated that slow freezing results in low survival and implantation rates, and can cause spindle abnormalities [8, 9]. These negative effects have limited the widespread use of slow freezing techniques. Since the invention of vitrification by Rall and Fahy [10], it has been widely applied for the cryopreservation of human oocytes [11], a variety of domestic and laboratory animals [12–21], as well as other mammalian embryos [21–30]. Furthermore, vitrification is considered to be a better alternative to slow freezing cryopreservation [31]. Regardless of the methodology used for cryopreservation, the pregnancy rate obtained with frozen/thawed gametes and embryos is still below that obtained with fresh gametes and embryos [32]. This finding suggests that improvements are still needed in oocyte and embryo cryopreservation. In this review, we address the major obstacles in the development of effective cryopreservation techniques to reveal which aspects require improvement and encourage further development in this field.

1 Types of cryoinjuries

During cryopreservation, mammalian oocytes and embryos can be damaged by various types of injuries [33]. For successful cryopreservation, conditions should be optimized to minimize injuries and maintain a high survival rate. The incidence of these injuries is closely related to the cryobiological properties of oocytes/embryos, such as sensitivity to chilling, permeability of the plasma membrane to water and cryoprotectant agents (CPAs), sensitivity to the chemical toxicity of the cryoprotectant, and tolerance for osmotic swelling and shrinkage. During cooling to subzero temperatures, cells may be exposed to several mechanisms of damage: as discussed below.

1.1 Chilling injury

Chilling injury usually occurs between +15 and –5°C. It induces partially irreversible changes in lipid droplets, lipid-rich membranes, and microtubuli of the mitotic or meiotic spindle [34,35]. Chilling injury is a common cryoinjury during application of slow freezing techniques, whereas vitrification involves a very high cooling rate and passages rapidly through this dangerous temperature zone, thus decreasing chilling injury to the oocytes and embryos [36]. Accordingly, vitrification is the only successful strategy for cryopreservation of intact porcine embryos containing extremely large amounts of chill-sensitive lipid droplets [37] and oocytes of various other species, which are sensitive to chilling, such as cattle, sheep, and horse [38].

1.2 Ice crystal formation

Ice crystal formation is considered to be the major source of injury [39] in the medium surrounding cells and inside the cells, including the cytoplasm and nucleus. It may occur between –5 and –80°C. In contrast to slow freezing, oocytes and embryos cryopreserved by vitrification are exposed to high concentrations of CPAs before immersion in liquid nitrogen at very high cooling rates (2000–20000°C min⁻¹) (Figure 1). Consequently, vitrification avoids ice crystal formation [39].

1.3 Fracture damage

Fracture damage occurs between –50 and –150°C, because of the mechanical effect of the solidified solution, especially in relatively large biological objects such as oocytes and embryos [40].

![Figure 1 Vitrification versus conventional slow freezing.](image-url)
1.4 Multiple aster formation

A newly discovered type of cryoinjury is multiple aster formation [41]. During vitrification, exposure of oocytes to highly concentrated CPAs and an ultrarapid cooling rate induces the formation of multiple asters near the male pronucleus. Thus, there is disruption to migration and development of pronuclei, resulting in delay in the first cleavage, and reduced potential for blastocyst formation [41]. A low concentration of glutathione in oocytes or low quality oocytes that cannot maintain the single sperm aster may be reasons for the increase of aster formation in vitrified oocytes. A recent study found that, high concentrations of glutathione in mature oocytes does not decrease the incidence of multiple aster formation after in vitro fertilization (IVF) of vitrified/warmed oocytes [42].

1.5 Osmotic stress

During cryopreservation of cells with high osmolar cryoprotectant solutions, the cells shrink immediately as water leaves in response to the difference in osmotic pressure between intracellular and extracellular solutions. It has been reported that water leaves a cell at about 5000 times faster than that of macromolecules and other solutes present in the cytoplasm [43]. Thawing is the reversal of the freezing process and is equally important. Because oocytes and embryos are more permeable to water than CPAs, frozen cells will swell or burst if they are placed directly in medium without CPAs after thawing. For this reason, a high concentration of non-permeating CPAs, such as sucrose, is usually used as an osmotic buffer to counteract the high concentration of CPAs in the cell. Changes in the cell shape can lead to cytoskeleton damage and fracture of the zona pellucida [44,45].

2 Cellular effects of cryopreservation on oocytes and embryos

2.1 Cytoskeleton

The oocyte cytoskeleton consists of three main components, microtubules (polymerized tubulin), microfilaments (polymerized actin), and intermediate filaments. During cryopreservation, at equilibration, osmotic shock may result in a shrunken and misshapen oocyte, which can damage the cytoskeleton. Exposure of oocytes to cooling [35], CPAs [46], or the freeze/thaw process [45] may cause microtubule depolymerization and DNA fragmentation [47], abnormal spindle configurations [48,49], chromosomal abnormalities [49], altered distribution or exocytosis of cortical granules [48], and cytoplasmic membrane fracture [50]. Similarly, after oocyte cryopreservation, there is a negative influence on microfilament functions [46]. These developmental perturbations can lead to abnormal distributions of mitochondria in the oolemma [51,52] and consequently result in reduced meiotic competence and fertilizability of oocytes, as well as developmental failure in the preimplantation embryo.

In oocytes, the meiotic spindles consist of microtubules that are constructed by polymerization of α- and β-tubulin. Meiotic spindles play vital roles in meiotic progression as well as chromosomal alignment and segregation [53]. Many technological advances have enabled visualization of the spindle. Two of these methods are confocal microscopy and polarized light microscopy. Confocal microscopy has limited value because it requires the oocyte to be stained, fixed, and nonviable [54]. Advances in polarized light microscopy have offered the opportunity to visualize the meiotic spindle non-invasively before and/or after cryopreservation [55, 56]. However, the inability of polarized light microscopy to distinguish between spindles with normal (bipolar) and highly disarranged conformation and to predict the degree of microtubule polymerization in metaphase II (MII) spindles of frozen/thawed oocytes make it an inefficient method to assess the MII spindle, especially after cryopreservation [57]. Recently, Gomes et al. [58] used polarized field microscopy, a noninvasive imaging method, and immunocytochemistry to compare the polymerization status of mouse oocyte spindles at various stages of meiosis, metaphase I (MI), telophase I (TI), and MII exposed to various temperatures (37°C, room temperature, 4°C, and vitrification) for 0, 10, 30, and 60 min. They found that the temperature- and time-dependent differences in the depolymerization/repolymerization equilibrium of oocyte spindles are related to the meiotic stage, in which TI shows less depolymerization at room temperature, 4°C, and after vitrification and warming than that of spindles in MI and MII oocytes.

Oocytes analyzed immediately after thawing display severe disorganization or disappearance of spindles following both slow freezing and vitrification methods [55,59,60] with a more deleterious effect of the slow freezing procedure [61]. However, there is disappearance and reappearance of meiotic spindles during MII after vitrification and slow freezing [35,61–63], which depends on the time interval after thawing, methods of freezing and thawing, and the species [55,59,60,64]. It has also been proposed that temperature-induced oocyte microtubule depolymerization may be dependent on the nuclear maturation state of oocytes [58,65]. A potential strategy to avoid spindle depolymerization is cryopreservation of oocytes at the germinal vesicle (GV) stage. However, immature oocytes are less permeable to water and CPAs [66], more sensitive to cryopreservation [67], and live births are rarer than those achieved with mature oocytes [68].

Cytokeratin is an intermediate filament that plays important roles in oocyte maturation and embryonic development [69]. The cytokeratin structure is affected during vitrification of both mature [70] and immature oocytes [71], which most likely contributes to oocyte death [70]. The post-warming survival and blastocyst formation rates ob-
tained after the use of cytochalasin B and taxol as cytoskeletal stabilizer agents during vitrification are still controversial. Improvement of the post-warming developmental competence of oocytes after using a cytoskeleton stabilizer has been reported in mouse [72], bovine [73], porcine [74, 75], and ovine [76] oocytes. However, some reports have indicated no improvements in bovine [77], porcine [78], and rabbit [79] oocytes. Further investigation is needed to overcome the consequences of cytoskeletal injuries and enhance cryopreservation procedures.

2.2 Zona pellucida

The zona pellucida is a glycoprotein membrane surrounding the plasma membrane of oocytes and preimplantation embryos. It is known to play a critical role in the entire fertilization process and blockade of polyspermy following initial penetration by one spermatozoon through triggering cortical granule exocytosis. The cortical reaction results in blockade of polyspermy by modifying the zona pellucida (zona reaction), oolemma, or both. During cryopreservation of oocytes, CPAs cause transient calcium increases in oocytes [80], and thus trigger cortical granule exocytosis [81] that is sufficient to cause zona hardening and compromises sperm penetration and fertilization [82]. A rapid change in the cell configuration is another negative effect of cryopreservation on oocytes. Alterations of the cell shape are observed as the cell folds in on itself, forming a concave appearance and thus resulting in fracture of the zona pellucida [44] and most likely contributing to polyspermic fertilization following oocyte cryopreservation.

2.3 Mitochondria

Mitochondria are the most abundant organelles in mammalian oocytes and their dysfunction or abnormalities are critical determinates of oocyte and embryonic developmental competence. Mitochondria are the sole source of energy production in the ooplasm to provide adenosine triphosphate (ATP) for fertilization and preimplantation embryonic development. A reduction in mitochondrial ATP production is associated with developmental failure in the preimplantation embryo [83]. Moreover, developmental failure in the preimplantation embryo may result from an abnormal distribution of mitochondria in the oolemma [52]. Vitrification has been reported to compromise mitochondrial function and reduce ATP content in human [84] and bovine [85] oocytes, which might contribute to poor oocyte development after cryopreservation [85]. The intracellular distribution of mitochondria is dependent on microtubules [86], which is important for redistribution of ATP and allows increased levels of ATP to be produced in different intracellular areas during periods of high energy requirements [86,87]. Cryopreservation has been reported to compromise the functions of microtubules [88], which can lead to abnormal distribution of mitochondria [51] and consequently alter intracellular ATP distribution. It has been suggested that the inability of mitochondria to return to normal distribution patterns can lead to less competent oocytes because an altered ATP distribution may affect vital processes during fertilization and development [51]. In addition, cryopreservation can lead to mitochondrial swelling [70,89], abnormally shaped mitochondria, and rupture of their inner and outer membranes [44,90]. To reduce the negative effect of vitrification on mitochondrial functions, addition of 1 mol L⁻¹ glycine to vitrification solutions results in maintenance of oocyte mitochondrial function and a subsequent improvement in the blastocyst developmental rate [51].

3 Molecular effects of cryopreservation

Cryopreservation has been reported to negatively affect the expression of genes related to oxidative stress, apoptosis, and the cell cycle as well as those important for the sperm-oocyte interaction [90–97]. Such alterations of gene expression might be responsible for the reduced ability of cryopreserved oocytes to undergo fertilization. Based on clinical results, the biological functions affected by slow freezing and vitrification are different with a more deleterious effect of the slow freezing procedure. Compared with vitrification, slow freezing results in down-regulation of genes involved in chromosomal structure maintenance and cell cycle regulation [98], poorer mRNA preservation (39.4%) in human MII (63.3%) [99], and a negative effect on protein expression and oocyte physiology [100]. Apoptosis is an underlying process in oocyte degeneration and embryo fragmentation [101]. Bcl2 family members play a major role in regulation of apoptosis and are considered as anti-apoptosis factors that promote cell survival, whereas BAX is a pro-apoptosis factor that promotes cell death [102]. Vitrification does not alter the expression pattern of BAX in canine oocytes or mouse embryos [90,103], whereas Bcl2 is strongly expressed in vitrified-warmed oocytes [90]. In contrast, vitrification has been reported to up-regulate pro-apoptotic genes (Fas, FasL, Bax, and Bcl-2) in bovine oocytes [93] and down-regulate Bcl2 in mouse embryos compared with that in the control [104].

CD9 is a four-transmembrane superfamily protein located on the plasma membrane of the mouse oocyte [105], and is essential for gamete fusion [106]. Lower CD9 mRNA expression has been observed in vitrified-warmed bovine [91] and ovine [94] oocytes compared with that in non-vitrified oocytes. There are a wide range of consequences resulting from vitrification of mouse embryos, including effects on metabolism and regulation of cellular and physiological activities such as proliferation, the cell cycle, development, biosynthesis, respiration, and stress-related gene expression [107,108]. Interestingly, vitrification causes ma-
jor changes in the gene expression of IVF bovine embryos, whereas no major changes are observed in the gene expression of in vivo-derived (IVV) embryos after vitrification [109]. A total of 268 genes are differentially regulated in IVF and IVV blastocysts, indicating greater sensitivity of IVF embryos to vitrification than that of IVV embryos [109].

There have been numerous studies on the epigenetic effects of vitrification [92,110–120]. Some studies have demonstrated that vitrification does not significantly alter gene methylation patterns in oocytes [110,115,119] and blastocysts [112]. In contrast, vitrification has been found to reduce gene methylation in mouse oocytes [113], embryos [112], and fetuses [117]. However, other studies have demonstrated that vitrification significantly increases gene methylation in mouse oocytes [116] as well as bovine two cell embryos and the resulting in vitro-derived blastocysts [111]. Regarding the effects of vitrification on acetylation patterns, several studies have indicated that vitrification significantly alters acetylation patterns in oocytes [92,114, 116,118,120]. The conclusions of these studies are somewhat contradictory, suggesting that the effects of vitrification on gene methylation patterns vary in a manner dependent on species, developmental stage, and genes, and may also depend on the size of the analyzed genomic regions [110]. The aberrant epigenetic modifications reported in these previous studies may partially explain the reduction in developmental competence of vitrified oocytes.

4 Cryopreservation of oocytes versus embryos
Regardless of the methodology used for cryopreservation, oocytes are much more difficult to cryopreserve than cleavage-stage embryos [121]. Attention should be paid to oocyte cryopreservation procedures because oocytes are particularly susceptible to cryodamage as discussed below.

4.1 Differences in size
It is well known that oocytes are the largest cell in the mammalian body. In cryobiology, the smaller the size of the sample, the better the cryopreservation results. The cumulative mass of cells decreases during the first week of embryonic development. At the expanded blastocyst stage, the mass may become as low as 1/10 to 1/100 of that of the oocyte. Consequently, because of the large surface area/volume ratio and low water permeability of oocytes, they are likely to retain water when frozen, creating intracellular ice that is extremely damaging to cells [39]. Importantly, the permeability of the plasma membrane of oocytes and embryos varies among maturational/developmental stages. For example, in bovine, the oocyte is less permeable to water and CPAs than that of the morula and blastocyst [66]. In mammalian oocytes/early embryos, water and CPAs move across the plasma membrane slowly by simple diffusion. Thus, long exposure to cryoprotectant solutions and a two-step treatment would be necessary to dehydrate the cell and allow CPAs to permeate sufficiently. In the morula and blastocyst, the movement of water and CPAs occurs rapidly via channels. Consequently, a one-step treatment and short exposure would be effective. Additionally, the temperatures at which oocytes are exposed to the CPA before cooling and at warming are critical. However, in the morula and blastocyst, the temperature may not be very important in terms of permeability, because facilitated diffusion through channels is less affected by temperature.

4.2 Differences in water content
Oocytes contain more water than embryos. However, the solution in the blastocoel may be a source for damage by ice crystal formation [122,123]. Such detrimental effects are more serious if they are present within oocytes.

4.3 Differences in cell number
Multicellular embryos can compensate for as much as a 50% loss of their cells as demonstrated by biopsies and bi-section of embryos. The oocyte has no such ability and cannot regenerate from a serious injury.

5 Attempts for successful cryopreservation
For development of a successful cryopreservation strategy, several attempts have been made to reduce cryoinjuries and maintain a high survival rate. The main results of these studies are summarized below.

5.1 Exposure to or equilibration of the cryoprotectants
To avoid ice crystal formation, much attention has been paid to the equilibration before cooling. There are two strategies are applied, the first is extremely short equilibration for both the diluted and concentrated CPAs [124] and the second is extended equilibration in the first diluted, followed by a short, but relatively prolonged incubation in the second concentrated cryoprotectant solution [125]. It has been reported that the shorter the time, the better the vitrification of oocytes and embryos [126]. In this regard, it has been suggested that the time should be less than 10 s [127]. On the other hand, a short equilibration time for oocytes in the vitrification solution results in low survival and blastocyst formation rates [128,129]. This observation suggests that, in the short protocol, intracellular water may not be completely replaced by CPAs in the oocytes after exposure to the vitrification solution, and may contribute to damage of some organelles inside the oocyte. However, a prolonged exposure time may ensure proper penetration of CPAs, providing appropriate protection for the entire oocyte.
5.2 The type of cryoprotectants

There is an obvious need for the use of highly permeable CPAs with low toxicity. Various substances are used for this purpose such as ethylene glycol (EG), glycerol, dimethylsulphoxide (DMSO), propylene glycol (PROH), and acetamide [130]. EG is the CPA of choice for this purpose [131]. It has been shown that DMSO facilitates the permeability of EG, which may have a beneficial effect on spindle polymerization and consequently a protective effect during oocyte vitrification [132]. EG is usually combined with DMSO to freeze and vitrify oocytes [39,133] and optimal concentrations have been studied in pigs [134]. However, vitrification of oocytes using the combination of EG and PROH provides better results than those using EG and DMSO in bovine [135] and humans [136]. This finding may be because of the lower toxicity of PROH than that of DMSO. Superior vitrification solutions with very low toxicity have been described and may have some advantages in reproductive cryobiology. For example, mouse ova vitrified with a solution known as 90% VM3 are able to be fertilized and develop to blastocysts at 80% of the rate of untreated control ova without the need for intracytoplasmic sperm injection [137]. The addition of low toxic, non-permeable CPAs in cryopreservation media is also required to facilitate dehydration and consequently minimize the toxic effects of a vitrification solution. For this purpose, trehalose and sucrose appear to be efficient [138]. They also counteract the osmotic effect exerted by CPAs on oocyte survival [21]. Methods to introduce and remove cryoprotectants also reduce toxicity and the resulting damage. During freezing, stepwise addition of CPAs or gradually increasing concentrations, as well as stepwise removal of these compounds upon warming/thawing help to minimize osmotic stress.

5.3 Avoiding zona pellucida hardening

Zona hardening and the subsequent low level of fertilization after oocyte cryopreservation [80] can be avoided by intracytoplasmic sperm injection [139], removal of calcium from the vitrification medium [81], and the use of bovine fetal serum [140].

5.4 Reducing chilling sensitivity of oocytes

The high sensitivity of oocytes to chilling injury because of large amounts of cytoplasmic lipid droplets [74] can be overcome by mechanical removal of lipid droplets [74,141], their reduction by chemical agents [142], and supplementation of culture media with L-carnitine that is known to play an essential role in fat metabolism [143–145].

5.5 Increasing cooling and warming rates by minimum volume methods

An extremely high cooling rate is one of the most important factors for improving the effectiveness of vitrification. Faster cooling and warming rates can be achieved by minimizing the volume of the solution in which oocytes/embryos are vitrified using minute tools such as electron microscope grids, open pulled straws (OPS), cryoloops, cryotops, hemi-straws, cryotips, and aluminum sheets. Successful development of oocytes into blastocysts (Table 1), pregnancy, and live births have been achieved by vitrification of mammalian oocytes (Table 2) and embryos (Table 3).

Table 1  Blastocyst rates of vitrified mammalian oocytes compared with that of fresh oocytes at GV/MII stages

| Species | Oocyte stage | Device used | Blastocyst % vs. control |
|---------|--------------|-------------|-------------------------|
| Sheep   | Immature oocytes | Cryoloop | 29.4 vs. 45.1 [12] |
| Cat     | In vitro matured oocytes | Cryotop | 10 vs. 25 [15] |
| Pig     | Immature (GV) & matured oocytes | Solid Surface Vitrification (SSV) | GV: 3 vs. 60; MII: 9 vs. 20 [18] |
| Buffalo | Denude immature oocytes | Solid Surface Vitrification (SSV) | 7.0 vs. 5.8 [19] |
| Buffalo | Denude immature oocytes | Cryoloop | 2.8 vs. 5.8 [19] |
| Mouse   | Matured oocytes | Nylon Loop | 83.9 vs. 84.1 [51] |
| Cattle  | Matured oocytes | Electron Microscope grids | 15 vs. 42 [124] |
| Human   | Matured oocytes | Cryotop | 48.7 vs. 47.5 [146] |
| Cat     | Immature oocytes | Cryotop | 1.6 vs. 34.4 [147] |
| Cattle  | Matured oocytes | Cryoloop | 36.7 vs. 55.2 [148] |

Table 2  Successful vitrification of mammalian oocytes by various tools

| Species | Oocyte stage | Device used | Clinical outcomes | References |
|---------|--------------|-------------|------------------|------------|
| Mouse   | In vivo matured oocytes | Cryotop | The offspring rate was 56.7% and 57.8 % for vitrified and fresh oocytes. | [13] |
| Cattle  | Immature oocytes | Aluminum Sheets and Nylon-Mesh holder | One calf was born from those vitrified by Aluminum Sheets. Live birth rate was 1.8%. | [14] |
| Cat     | In vitro matured oocytes | Cryotop | Four live kittens were born, live birth was 10%. | [15] |
| Human   | In vitro matured oocytes | Cryoleaf | A woman delivered a single healthy live baby, live birth rate was 5.8%. | [149] |
### Table 3 Successful vitrification of mammalian embryos by various tools

| Species | Embryo stage | Device used | Clinical outcomes | References |
|---------|--------------|-------------|-------------------|------------|
| Sika deer (Cervus nippon) | Eight-cell stage, morula and blastocyst | 0.25 mL plastic straw | Birth rates were 64.3% and 53.9% for fresh and vitrified embryos. | [23] |
| Goat | Morula and blastocysts. | OPS | When used 40% (v/v) EG or 15% (v/v) EG+15% (v/v) DMSO in vitrification solution, the kidding rates were 46.2%, 51.4%. these rates were no significantly different from that of fresh ones 57.1%. | [25] |
| European polecat (Mustela putorius) | Morula and blastocyst | OPS | Two recipients delivered a total of eight offspring (16% survival rate). | [26] |
| Cattle | Blastocyst produced by Somatic cell nuclear transfer | 0.25 ml plastic straw | Two healthy calves (25%) were obtained from fresh blastocysts and one (11%) from vitrified/thawed blastocysts. | [27] |
| Sheep | In vivo derived embryos | OPS | The lambing rates were 56%. | [28] |
| Rabbit | Morula and blastocysts. | Modified (sealed) OPS | Vitrified embryos resulted in 51.7% live birth compared to 58.5% for fresh embryos. | [29] |
| Human | Blastocyst | Electron Microscope grids | 34.1% clinical pregnancy and 11 live births were achieved. | [30] |
| Human | Blastocyst | Cryoloop | 23 healthy babies were born in 18 deliveries, and 37 pregnancies were ongoing. | [150] |
| Human | Blastocyst | Hemi-straw | 27% ongoing pregnancy rate was obtained. | [151] |
| Pig | Blastocyst | OPS | Nine recipients came to term (42.9%) and farrowed an average of 5.4 ± 0.8 piglets (range from 3 to 9). | [152] |

using various carrier systems.

### 6 Conclusion and future perspectives

Significant advances in cryopreservation procedures are clearly evident when comparing current results with those obtained when the technology first became available. The methods to cryopreserve mammalian oocytes/embryos can be divided into two categories, slow freezing and vitrification. It is evident from data summarized in this review that vitrification is a viable approach for broad application of cryopreservation in many areas of ART. For humans, improvements in embryo cryopreservation will allow transfer of fewer embryos, decreasing the incidence of multiple pregnancies, which is a major complication of ART treatment. With a better understanding of the physical and biological principles of vitrification, we can achieve more success and higher efficiency. Several aspects should be taken into account during cryopreservation of oocytes and embryos as follows.

#### 6.1 Plasma membrane permeability

The permeability of the plasma membrane of oocytes and embryos varies among maturational/developmental stages, even in the same species. Therefore, the survival of oocytes/embryos after cryopreservation differs using same cryopreservation protocol.

#### 6.2 Methodology of cryopreservation

Maximizing the survival rate of oocytes/embryos subjected to freezing and thawing requires careful selection of less toxic cryoprotective agents, close monitoring of their temperature, time of exposure, concentration, and their stepwise addition and removal from cells. Vitrification solutions based on minimal perturbation of intracellular water appear to be superior and promote successful vitrification of mammalian oocytes and embryos.

#### 6.3 Use of aseptic technologies

The high risk of contamination because of the direct contact of oocytes/embryos with liquid nitrogen [153] using open carriers raises the need to develop safety strategies to reduce the risk of contamination. Recently, satisfactory results obtained using various types of “closed” systems [22,154] have allowed the transition to closed and more safe vitrification systems. These findings highlight the need for further efforts to optimize protocols for closed vitrification systems.

Further studies are needed to ensure the safest and most expeditious development of oocyte/embryo cryopreservation technology and advance vitrification technology to achieve undamaged oocytes/embryos after cryopreservation. Such studies will involve continued molecular and biochemical evaluation of various CPAs and careful selection of the most effective CPAs combined with efficient storage methods. These advances will undoubtedly have a significant effect on the practical use of cryopreservation as a major part in ART.

This review was supported by grants from the National High Technology Research and Development Program of China (2011AA100602), the National Science and Technology Major Project of China (2013ZX08007-004, 2013ZX08008-004), and the National Natural Science Foundation of China (31371486).

1. Chang M. The effects of low temperature on fertilized rabbit ova in vitro, and the normal development of ova kept at low temperature for several days. J Gen Physiol, 1948; 31: 385–410
2 Chang M. Fertilizability of rabbit ova and the effects of temperature in vitro on their subsequent fertilization and activation in vivo. J Exp Zool Exp Zool, 1952,121: 351–381
3 Whittingham D. Fertilization in vitro and development to term of unfertilized mouse oocytes previously stored at –96°C. J Reprod Fertil, 1977, 49: 89–94
4 Chen C. Pregnancy after human oocyte cryopreservation. Lancet, 1986, 327: 884–886
5 Al-Hassani S, Kirsch J, Diedrich K, Blanke S, Van der Ven H, Krebs D. Successful embryo transfer of cryopreserved and in vitro fertilized rabbit oocytes. Hum Reprod, 1989, 4: 77–79
6 Fuku E, Kojima T, Shioya Y, Marcus G, Downey B. In vitro fertilization and development of frozen-thawed bovine oocytes. Cryobiology, 1992, 29: 485–492
7 Macellani L, Carnevalle E, Coutinho da Silva M, Scoggin C, Bruenmer J, Squires E. Fertilizations from vitrified equine oocytes collected from super-stimulated and non-stimulated mares. Theriogenology, 2002, 58: 911–919
8 Oktay K, Cil AP, Bang H. Efficiency of oocyte cryopreservation: a meta-analysis. Fertil Steril, 2006, 86: 70–80
9 Bromfied J, Coticchio G, Hutt K, Scijano R, Borini A, Albertini D. Meiotic spindle dynamics in human oocytes following slow-cooling cryopreservation. Hum Reprod, 2009, 24: 2114–2123
10 Rall W, Fady G. Ice-free cryopreservation of mouse embryos at –96°C by vitrification. Nature, 1985, 313: 573–575
11 Smith GD, Serafini PC, Fioravanti J, Yadid I, Coslovsky M, Hassun P, Alegretti JR, Motta EL. Prospective randomized comparison of human oocyte cryopreservation with slow-rate freezing or vitrification. Fertil Steril, 2010, 94: 2088–2095
12 Mouawad AR, Zhu J, Choi I, Amarnath D, Chen W, Campbell KH. Production of good-quality blastocyst embryos following IVF of oocytes vitrified at the germinal vesicle stage using a cryoloop. Reprod Fert Develop, 2013, 25: 1204–1215
13 Kohaya N, Fujiwara K, Ito J, Kashiwazaki N. Generation of Live Offspring from Vitrified Mouse Oocytes of C57BL/6 Strain. PLoS One, 2013, 8: e58063
14 Aono A, Nagatomo H, Takuma T, Nonaka R, Ono Y, Wada Y, Abe Y, Takahashi M, Watanabe T, Kawahara M. Dynamics of intracellular phospholipid membrane organization during oocyte maturation and successful vitrification of immature oocytes retrieved by ovum pick-up in cattle. Theriogenology, 2013, 79: 1146–1152
15 Pope CE, Gómez MC, Kagawa N, Kuwayama M, Leibo SP, Dresser BL. In vivo survival of domestic cat oocytes after vitrification, intracytoplasmic sperm injection and embryo transfer. Theriogenology, 2012, 77: 531–538
16 de Leon PMM, Campos VE, Corcini CD, Santos ECS, Rambo G, Lucía Jr T, Deschamps JC, Collares T. Cryopreservation of immature equine oocytes, comparing a solid surface vitrification process with intracytoplasmic sperm injection: a novel, paired randomized controlled trial using DNA fingerprinting. Fertil Steril, 2012, 98: 644–649
17 Vieira A, Forell F, Feltrin C, Rodrigues J. Open pulled straws and the use of a synthetic ice blocker. Theriogenology, 2012, 78: 1627–1632
18 Paragotidus Y, Vanderzwalmen P, Prapas Y, Kasapi E, Goudakou M, Papatheodorou A, Passadaki T, Petousis S, Nikolettos N, Veletza S, Prapas N, Maroulis G. Open versus cooled vitrification of blastocysts from an oocyte-donation programme: a prospective randomized study. Reprod Biomed Online, 2013, 26: 470–476
19 Wang L, Zhou G, Shi W, Shi J, Tian X, Gao C, Zhang L, Zhu SE, Zhang TT, Zeng SM, Liu GS. First live offspring born in superovulated sika deer (Cervus nippon) after embryo vitrification. Theriogenology, 2012, 78: 1627–1632
20 Nagashima H, Hiruma K, Saito H, Tomi R, Ueno S, Nakayama N, Matsumaru H, Kurome M. Production of live piglets following cryopreservation of embryos derived from in vitro-matured oocytes. Biol Reprod, 2007, 76: 900–905
21 Hong QH, Tian SJ, Zhu SE, Feng JZ, Yan CL, Zhao XM, Liu GS, Zheng SM. Vitrification of boer goat morulae and early blastocysts by straw and open-pulled straw method. Reprod Domest Anim, 2007, 42: 34–38
22 Piltti K, Lindeberg H, Aalto J, Korhonen H. Live births after transfer of OPS vitrified-warmed embryos in the farmed European polecat (Mustela putorius). Theriogenology, 2004, 61: 811–820
23 Gong G, Dai Y, Fan B, Zhu H, Zhu S, Wang H, Wang L, Tang B, Li R, Wen R, Liu Y, Huang Y, Zhang L, Sun X, Li N. Birth of calves expressing the enhanced green fluorescent protein after transfer of fresh or vitrified/thawed blastocysts produced by somatic cell nuclear transfer. Mol Reprod Dev, 2004, 69: 278–288
24 Isachenko V, Alabart JL, Dattena M, Nawroth F, Cappai P, Isachenko E, Cocero MJ, Olivera J, Roche A, Accardo C, Krivokharchenko A, Folch J. New technology for vitrification and field (micro-freeze) warming and transfer of small ruminant embryos. Theriogenology, 2003, 59: 1209–1218
25 López-Béjar M, López-Gatius F. Nonequilibrium cryopreservation of rabbit embryos using a modified (sealed) open pulled straw procedure. Theriogenology, 2002, 58: 1541–1552
26 Cho HJ, Son WY, Yoon SH, Lee SW, Lim JH. An improved protocol for dilution of cryoprotectants from vitrified human blastocysts. Hum Reprod, 2002, 17: 2419–2422
27 Kuleshova LL, Lopata A. Vitrification can be more favorable than slow cooling. Fertil Steril, 2002, 78: 449–454
28 Forman EI, Li X, Ferry KM, Scott K, Treff NR, Scott RT. Oocyte vitrification does not increase the risk of embryonic aneuploidy and diminish the implantation potential of blastocysts created after intracytoplasmic sperm injection: a novel, paired randomized controlled trial using DNA fingerprinting. Fertil Steril, 2012, 98: 644–649
29 Kasai M. Advances in the cryopreservation of mammalian oocytes and embryos: development of ultrarapid vitrification. Reprod Med Biol, 2002, 1: 1–9
30 Martino A, Pollard JW, Leibo S. Effect of chilling bovine oocytes on their developmental competence. Mol Reprod Dev, 1996, 45: 503–512
31 Aman RR, Parks JE. Effects of cooling and rewarming on the meiotic spindle and chromosomes of in vitro-matured bovine oocytes. Biol Reprod, 1994, 50: 103–110
32 Rall W. Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiology, 1987, 24: 387–402
33 Berthollet F, Martinat-Botté F, Perreaux C, Terqui M. Birth of pigs after OPS vitrification and transfer of compacted morula stage embryos with intact zona pellucida. Reprod Nutr Dev, 2001, 41: 267
34 Ledda S, Bologliolo L, Succu S, Ariu F, Bebbere D, Leoni GG, Naitana A, Alabart JL, Dattena M, Nawroth F, Cappai P, Isachenko E, Cocero MJ, Olivera J, Roche A, Accardo C, Krivokharchenko A, Folch J. New technology for vitrification and field (micro-freeze) warming and transfer of small ruminant embryos. Theriogenology, 2003, 59: 1209–1218
35 Aman RR, Parks JE. Effects of cooling and rewarming on the meiotic spindle and chromosomes of in vitro-matured bovine oocytes. Biol Reprod, 1994, 50: 103–110
36 Rall W. Factors affecting the survival of mouse embryos cryopreserved by vitrification. Cryobiology, 1987, 24: 387–402
bovine oocytes after in vitro fertilization. Theriogenology, 2012, 77: 908–915
42 Har a Y, Yamane I, Noto I, Kagawa N, Kuwayama M, Hirabayashi M, H ochi S. Microtubule assembly and in vitro development of bovine oocytes with increased intracellular glutathione level prior to vitrification and in vitro fertilization. Zygote, 2013, 26: 1–7
43 Jackowski S, Leibo S, Mazur P. Glycerol permeabilities of fertilized and unfertilized mouse ova. J Exp Zool, 1980, 212: 329–341
44 Wu C, Rui R, Dai J, Zhang C, Ju S, Xie B, Lu X. Zheng X. Effects of cryopreservation on the developmental competence, ultrastructure and cytoskeletal structure of porcine oocytes. Mol Reprod Dev, 2006, 73: 1454–1462
45 Aigner S, Van der Elst J, Siebzehnrubl E, Wildt L, Lang N, Van Steirteghem A. The influence of slow and ultra-rapid freezing on the organization of the meiotic spindle of the mouse oocyte. Hum Reprod, 1992, 7: 857–864
46 Vincent C, Garnier V, Heyman Y, Renard J. Solvent effects on cytoskeletal organization and in vitro survival after freezing of rabbit oocytes. J Reprod Fertil, 1989, 87: 809–820
47 Sharma GT, Dubey PK, Chandra V. Morphological changes, DNA damage and developmental competence of in vitro matured, vitrified-thawed buffalo (Bubalus bubalis) oocytes: A comparative study of two cryoprotectants and two cryodevices. Cryobiology, 2010, 60: 315–321
48 Morató R, Megas T, Maddox-Hyttel P. Ultrastructure of bovine oocytes exposed to Taxol prior to OPS vitrification. Mol Reprod Dev, 2008, 75: 1318–1326
49 Boiso I, Marti M, Santaló J, Ponsà M, Barri PN, Veiga A. A confocal microscopy analysis of the spindle and chromosome configurations of human oocytes cryopreserved at the germinal vesicle and metaphase II stage. Hum Reprod, 2002, 17: 1885–1891
50 Zhou GB, Li N. Cryopreservation of porcine oocytes: recent advances. Mol Hum Reprod, 2009, 15: 279–285
51 Zander-Fox D, Cashman KS, Lane M. The presence of 1 mM glycine in vitrification solutions protects oocyte mitochondrial homeostasis and improves blastocyst development. J Assist Reprod Genet, 2013, 30: 107–116
52 Nagai S, Mabuchi T, Hirata S, Shoda T, Kasai T, Yokota S, Shitara H, Yonekawa H, Hoshi K. Correlation of abnormal mitochondrial distribution in mouse oocytes with reduced developmental competence. Tohoku J Exp Med, 2006, 207: 137–144
53 Schatten G, Simmerly C, Schatten H. Microtubule configurations during fertilization, mitosis, and early development in the mouse and the requirement for egg microtubule-mediated motility during mammalian fertilization. Proc Natl Acad Sci USA, 1985, 82: 4152–4156
54 Rienzi L, Ubaldi F, Iacobelli M, Minasi MG, Romano S, Greco E. Meiotic spindle visualization in living human oocytes. Reprod Biomed Online, 2005, 10: 192–198
55 Rienzi L, Martinez F, Ubaldi F, Minasi M, Iacobelli M, Tesarik J, Greco E. Polysome analysis of meiotic spindle changes in living metaphase II human oocytes during the freezing and thawing procedures. Hum Reprod, 2004, 19: 655–659
56 Bianchi V, Coticchio G, Fava L, Flamigni C, Borini A. Meiotic spindle imaging in human oocytes frozen with a slow freezing procedure involving high sucrose concentration. Hum Reprod, 2005, 20: 1078–1083
57 Coticchio G, Scaino R, Hutt K, Bromfield J, Borini A, Albertini DF. Comparative analysis of the metaphase II spindle of human oocytes through polarized light and high-performance confocal microscopy. Fertil Steril, 2010, 93: 2056–2064
58 Gomes C, Merlini M, Konheim J, Serafini P, Motta EL, Baracat EC, Smith GD. Oocyte meiotic-stage-specific differences in spindle depolymerization in response to temperature changes monitored with polarized field microscopy and immunocytochemistry. Fertil Steril, 2012, 97: 714–719
59 Eroglu A, Toth TL, Toner M. Alterations of the cytoskeleton and polyplody induced by cryopreservation of metaphase II mouse oocytes. Fertil Steril, 1998, 69: 944–957
60 Chen SU, Lien YR, Chen HF, Chao KH, Ho HN, Yang YS. Open pulled straws for vitrification of mature mouse oocytes preserve patterns of meiotic spindles and chromosomes better than conventional straws. Hum Reprod, 2000, 15: 2598–2603
61 Martinez-Burgos M, Herrero L, Megías D, Salvanes R, Montoya MC, Cobo AC, Garcia-Velasco JA. Vitrification versus slow freezing of oocytes: effects on morphologic appearance, meiotic spindle configuration, and DNA damage. Fertil Steril, 2011, 95: 374–377
62 Ciotti PM, Porcu E, Notarangelo L, Magrini O, Bazzocchi A, Venturoli S. Meiotic spindle recovery is faster in vitrification of human oocytes compared to slow freezing. Fertil Steril, 2009, 91: 2399–2407
63 Chang CC, Lin CJ, Sung LY, Kort HI, Tian XC, Nagy ZP. Impact of phase transition on the mouse oocyte spindle during vitrification. Reprod Biomed Online, 2011, 22: 184–191
64 Gook DA, Osborn SM, Johnston W. Cryopreservation of mouse and human oocytes using 1,2-propanediol and the configuration of the meiotic spindle. Hum Reprod, 1993, 8: 1101–1109
65 Goud A, Goud P, Qian C, Van der Elst J, Van Maele G, Dhont M. Cryopreservation of human germinal vesicle stage and in vitro matured M II oocytes: influence of cryopreservation media on the survival, fertilization, and early cleavage divisions. Fertil Steril, 2000, 74: 487–494
66 Acea Y, Liu J, Peter A, Crisler E, Crisler J. Effect of developmental stage on bovine oocyte plasma membrane water and cryoprotectant permeability characteristics. Mol Reprod Dev, 1998, 49: 408–415
67 Ghetter Y, Yavin S, Shalgi R, Arav A. The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. Hum Reprod, 2005, 20: 3385–3389
68 Tucker MJ, Wright G, Morton PC, Massey JB. Birth after cryopreservation of immature oocytes with subsequent in vitro maturation. Fertil Steril, 1998, 70: 578–579
69 Ian Gallicano G, McGaughey RW, Capco DG. Cytoskeleton of the mouse egg and embryo: reorganization of planar elements. Cell Motil Cytoskeleton, 1991, 18: 143–154
70 Valojerdi MR, Salehnia M. Developmental potential and ultrastructural injuries of metaphase II (MII) mouse oocytes after slow freezing or vitrification. J Assist Reprod Genet, 2005, 22: 119–127
71 Wei X, Xiangwei F, Guangbin Z, Jing X, Liang W, Ming D, Dianshui Y, Mingsing Y, Jianhui T, Shien Z. Cytokeratin distribution and expression during the maturation of mouse germinal vesicle oocytes after vitrification. Cryobiology, 2013, 66: 261–266
72 Park SE, Chung HM, Cha KY, Hwang WS, Lee ES, Lim JM. Cryopreservation of ICR mouse oocytes: improved post-thawed preimplantation development after vitrification using Taxol, a cytoskeleton stabilizer. Fertil Steril, 2001, 75: 1177–1184
73 Schmidt D, Nedambale T, Kim C, Maier D, Yang X, Tian X. Effect of cytoskeleton stabilizing agents on bovine matured oocytes following vitrification. Fertil Steril, 2004, 82: S26–S
74 Ogawa B, Ueno S, Nakayama N, Matsunari H, Nakano K, Fujiwara T, Ikezawa Y, Nagashima H. Developmental ability of porcine in vitro matured oocytes at the meiosis II stage after vitrification. J Reprod Dev, 2010, 56: 356–361
75 Shi WQ, Zhu SE, Zhang D, Wang WH, Tang GL, Hou YP, Tian SJ. Improved development by Taxol pretreatment after vitrification of in vitro matured porcine oocytes. Reproduction, 2006, 131: 795–804
76 Zhang J, Nedambale T, Yang M, Li J. Improved development of ovine matured oocyte following solid surface vitrification (SSV): Effect of cumulus cells and cytoskeleton stabilizer. Anim Reprod Sci, 2009, 110: 46–55
77 Mezzalira A, Vieira A, Barbieri D, Machado M, Thaler Neto A, Bernardi M. Cryopreservation/cryobiology-vitrification of matured
bovine oocytes treated with Cytochalasin B. Theriogenology, 2002, 57: 472

78 Fujihira T, Nagai H, Fukui Y. Relationship between equilibration times and the presence of cumulus cells, and effect of Taxol treatment for vitrification of in vitro matured porcine oocytes. Cryobiology, 2005, 51: 339–343

79 Jiménez-Trigos E, Naturil-Alfonso C, Vicente JS, Marco-Jiménez F. Post-warming competence of in vitro matured rabbit oocytes treated with cytoskeletal stabilization (Taxol) and cytoskeletal relaxant (Cytochalasin B) before vitrification. Reprod Domest Anim, 2013, 48: 15–19

80 Larman MG, Sheehan CB, Gardner DK. Calcium-free vitrification reduces cryoprotectant-induced zona pellucida hardening and increases fertilization rates in mouse oocytes. Reproduction, 2006, 131: 53–61

81 Kline D, Kline JT. Repetitive calcium transients and the role of calcium in exocytosis and cell cycle activation in the mouse egg. Dev Biol, 1992, 149: 80–89

82 Pickering SJ, Braude PR, Johnson ML. Cryoprotection of human oocytes: inappropriate exposure to DMSO reduces fertilization rates. Hum Reprod, 1991, 6: 142–143

83 Brevini TA, Vassena R, Francisci C, Gandolfi F. Role of adenosine triphosphate, active mitochondria, and microtubules in the acquisition of developmental competence of parthenogenetically activated pig oocytes. Biol Reprod, 2005, 72: 1218–1223

84 Manipalviratn S, Tong ZB, Stegmann B, Widra E, Carter J, DeCherney A. Effect of vitrification and thawing on human oocyte follicular maturation and gene expression. Mol Reprod Dev, 2006, 73: 1380–1390

85 Zhao XM, Du WH, Wang D, Hao HS, Liu Y, Qin T, Zhu HB. Effect of cyclosporine pretreatment on mitochondrial function in vitrified bovine mature oocytes. Fertil Steril, 2011, 95: 2786–2788

86 van Blerkom J. Mitochondria in human oogenesis and preimplantation embryogenesis: engines of metabolism, ionic regulation and developmental competence. Reproduction, 2004, 128: 269–280

87 Cummins J. The role of mitochondria in the establishment of oocyte functional competence. Eur J Obstet Gynecol Reprod Biol, 2011, 155: 236–242

88 Cobo A, Perez S, De los Santos M, Zulategui J, Roman K, Remohi J. Effect of different cryopreservation protocols on the metaphase II spindle in human oocytes. Reprod Biomed Online, 2008, 17: 350–359

89 Hochi S, Kozawa M, Fujimoto T, Honda E, Yamada J, Oguri N. In vitro maturation and transmission electron microscopic observation of horse oocytes after vitrification. Cryobiology, 1996, 33: 300–310

90 Tarathum B, Saikhun K, Sangsuwan P, Kitiyanyant E. Effects of vitrification on nuclear maturation, ultrastructural changes and gene expression of canine oocytes. Reprod Biol Endocrinol, 2010, 8: 70

91 Zhou GB, Zeng Y, Meng QG, Liu Y, Dai YP, Zou SE, Bunch TD, Hou YP. Decreased expression of CD9 in bovine oocytes after cryopreservation and the relationship to fertilization capacity. Mol Reprod Dev, 2013, 80: 451–459

92 Li J, Fu X, Mo X, Yue M, Jia B, Zhu S. Vitrification alters acH4K12 and acH4K16 levels in sheep oocytes at various developmental stages. Small Ruminant Res, 2013, 112: 108–113

93 Anchamparuthy VM, Pearson RE, Gwazdauskas FC. Expression pattern of apoptotic genes in vitrified-thawed bovine oocytes. Reprod Domest Anim, 2010, 45: 83–90

94 Saccu S, Bubbere D, Bogliolo L, Arien F, Fois S, Leoni GG, Bartlinguer F, Natania S, Ledda S. Vitrification of in vitro matured ovine oocytes affects in vitro pre-implantation development and mRNA abundance. Mol Reprod Dev, 2008, 75: 538–546

95 Lee RK-K, Li SH, Lu CH, Ho HY, Chen YJ, Yeh HI. Abnormally low expression of connexin 37 and connexin 43 in subcutaneously transplanted cryopreserved mouse ovarian tissue. J Assist Reprod Genet, 2008, 25: 489–497

96 Wen Y, Quintero R, Chen B, Shu Y, Polan ML, Behr B. Expression of CD9 in frozen-thawed mouse oocytes: preliminary experience. Fertil Steril, 2007, 88: 526–529

97 Liu HC, He Z, Rosenwaks Z. Mouse ovarian tissue cryopreservation has only a minor effect on in vitro follicular maturation and gene expression. J Assist Reprod Genet, 2003, 20: 421–431

98 Monzo C, Haozzi D, Roman K, Assou S, Dechau H, Hamamah S. Slow freezing and vitrification differentially modify the gene expression profile of human metaphase II oocytes. Hum Reprod, 2012, 27: 2160–2168

99 Chamayou S, Bonaventura G, Allecci C, Tibullo D, Di Raimondo F, Guglielmino A, Barcellona ML. Consequences of metaphase II oocyte cryopreservation on mRNA content. Cryobiology, 2011, 62: 130–134

100 Larman M, Katz-Jaffe M, Sheehan C, Gardner D. 1,2-propanediol and the type of cryopreservation procedure adversely affect mouse oocyte physiology. Hum Reprod, 2007, 22: 250–259

101 Brenner C, Exley G, Allikmäe K, Cohen J, McElhinny A, Stachecki J, Warner CM. Apoptosis and human embryo survival. Fertil Steril, 1997, 68(S86): 174

102 Yang MY, Rajamahendran R. Expression of Bel-2 and Bax proteins in relation to quality of bovine oocytes and embryos produced in vitro. Anim Reprod Sci, 2002, 70: 159–169

103 Dhali A, Anchamparuthy VM, Butler SP, Pearson RE, Mullarky IK, Gwazdauskas FC. Effect of droplet vitrification on development competence, actin cytoskeletal integrity and gene expression in in vitro cultured mouse embryos. Theriogenology, 2009, 71: 1408–1416

104 Dhali A, Anchamparuthy VM, Butler SP, Pearson RE, Mullarky IK, Gwazdauskas FC. Gene expression and development of mouse zygotes following droplet vitrification. Theriogenology, 2007, 68: 1292–1298

105 Barrau-Lange V, Boissonnas CC, Serres C, Auer J, Schmitt A, Lefèvre B, Wolf JP, Ziyat A. Membrane transfer from oocyte to sperm occurs in two CD9-independent ways that do not supply the fertilising ability of CD9-deleted oocytes. Reproduction, 2012, 144: 53–66

106 Miyado K, Yoshida K, Yamagata K, Sakakibara K, Okabe M, Wang X, Miymoto K, Akutsu H, Kondo T, Takahashi Y, Ban T, Ito C, Toshimori K, Nakamura A, Ito M, Miyado K, Mekada E, Umezawa A. The fused ability of sperm is bestowed by CD9-containing vesicles released from eggs in mice. Proc Natl Acad Sci USA, 2008, 105: 12921–12926

107 Mamo S, Bodo S, Kobolak J, Polgar Z, Tolygessy G, Dinneyes A. Gene expression profiles of vitrified in vivo derived 8-cell stage mouse embryos detected by high density oligonucleotide microarrays. Mol Reprod Dev, 2006, 73: 1380–1392

108 Boonkuso D, Gal AB, Bodo S, Gorhony B, Kitiyanyant Y, Dinneyes A. Gene expression profiles and in vitro development following vitrification of pronuclear and 8-cell stage mouse embryos. Mol Reprod Dev, 2006, 73: 700–708

109 Aksu DA, Agea C, Aksu S, Bagis H, Akkou T, Caputcu AT, Arat S, Taskin AC, Kizil SH, Karasahin T, Akyol N, Satilmis M, Sagirkaya H, Ustuner B, Nur Z, Agea Y. Gene expression profiles of vitrified in vitro- and in vivo-derived bovine blastocysts. Mol Reprod Dev, 2012, 79: 613–625

110 Zhao XM, Ren JJ, Du WH, Hao HS, Wang D, Qin T, Liu Y, Zhu HB. Effect of vitrification on promoter Cpg island methylation patterns and expression levels of DNA methyltransferase 1o, histone acetyltransferase 1, and deacetylase 1 in metaphase II mouse oocytes. Fertil Steril, 2013, 100: 256–261

111 Zhao XM, Ren JJ, Du WH, Hao HS, Wang D, Liu Y, Qin T, Zhu HB. Effect of 5-aza-2′-deoxycytidine on methylation of the putative imprint control region of H19 during the in vitro development of vitrified bovine two-cell embryos. Fertil Steril, 2012, 98: 222–227

112 Zhao XM, Du WH, Hao HS, Wang D, Qin T, Liu Y, Zhu HB. Effect of vitrification on promoter methylation and the expression of pluripotency and differentiation genes in mouse blastocysts. Mol Reprod Dev, 2012, 79: 445–450
Moussa M, et al. Sci China Life Sci September (2014) Vol.57 No.9

113 Milroy C, Liu L, Hammond S, Hammond A, Peterson CM, Carrell DT. Differential methylation of pluripotency gene promoters in in vitro matured and vitrified, in vivo-matured mouse oocytes. Fertil Steril, 2011, 95: 2094–2099

114 Li JJ, Pei Y, Zhou GB, Suo L, Wang YP, Wu QQ, Fu XW, Hou YP, Zhu SE. Histone deacetyltransferase1 expression in mouse oocyte and their in vitro-fertilized embryo: effect of oocyte vitrification. CryoLetters, 2011, 32: 13–20

115 Al-Khtib M, Perret A, Khoury R, Ibalah-Romdhane S, Blachère T, Greze C, Lornage J, Lefèvre A. Vitrification at the germline vesicle stage does not affect the methylation profile of H19 and KCNQ1OT1 imprinting centers in human oocytes subsequently matured in vitro. Fertil Steril, 2011, 95: 1955–1960

116 Yan LY, Yan J, Qiao J, Zhao PL, Liu P. Effects of oocyte vitrification on histone modifications. Reprod Fert Develop, 2010, 22: 920–925

117 Wang Z, Xu L, He F. Embryo vitrification affects the methylation of the H19/Igf2 differentially methylated domain and the expression of H19 and Igf2. Fertil Steril, 2010, 93: 2729–2733

118 Suo L, Meng Q, Pei Y, Fu X, Wang Y, Bunch TD, Zhu S. Effect of cryopreservation on acetylation patterns of lysine 12 of histone H4 (acH4K12) in mouse oocytes and zygotes. J Assist Reprod Genet, 2010, 27: 735–741

119 Trapphoff T, El Hajj N, Zechner U, Haaf T, Eichenlaub-Ritter U. DNA integrity, growth pattern, spindle formation, chromosomal constitution and imprinting patterns of mouse oocytes from vitrified pre-antral follicles. Hum Reprod, 2010, 25: 3025–3042

120 Spinacci M, Vallorani C, Bucci D, Tamanini C, Porcu E, Galeati G. Vitrification of pig oocytes induces changes in histone H4 acetylation and histone H3 lysine 9 methylation (H3K9). Vet Res Commun, 2012, 36: 165–171

121 Prentice JR, Anzar M. Cryopreservation of mammalian oocyte for conservation of animal genetics. Vet Med Int, 2011, doi: 10.4061/2011/146405

122 Son WY, Yoon SH, Yoon HJ, Lee SM, Lim JH. Pregnancy outcome following transfer of human blastocysts vitrified on electron microscopy grids after induced collapse of the blastocoele. Hum Reprod, 2003, 18: 137–139

123 Hiraoka K, Hiraoka K, Kinutani M, Kinutani K. Blastocoele collapse by microspotiing prior to vitrification gives excellent survival and pregnancy outcomes for human day 5 and 6 expanded blastocysts. Hum Reprod, 2004, 19: 2884–2888

124 Martino A, Pollard JW, Leibo S. Effect of chilling bovine oocytes on their developmental competence. Mol Reprod Dev, 1996, 45: 503–512

125 Dinnyés A, Dai Y, Jiang S, Yang X. High developmental rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. Biopol Reprod, 2000, 63: 513–518

126 Fahy GM, Lilley TH, Lindsell D, Rossini MSJ, Meryman HT. Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms. Cryobiology, 1990, 27: 247–268

127 Nakagata N. High survival rate of unfertilized mouse oocytes after vitrification. J Reprod Fertil, 1989, 87: 479–483

128 Wang CT, Liang L, Witz C, Williams D, Griffith J, Skorupski J, Haddad G, Gill J, Wang W. Optimized protocol for cryopreservation of human eggs improves developmental competence and implantation of resulting embryos. J Ovarian Res, 2013, 6: 15

129 Wang L, Liu J, Zhou GB, Hou YP, Li JH, Zhu SE. Quantitative investigations on the effects of exposure durations to the combined cryoprotective agents on mouse oocyte vitrification procedures. Biol Reprod, 2011, 85: 884–894

130 Shaw J, Jones G. Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. Hum Reprod Update, 2003, 9: 583–605

131 Tan VJ, Xiong Y, Ding GL, Zhang D, Meng Y, Huang HF, Sheng JZ. Cryoprotectants up-regulate expression of mouse oocyte AQP7, which facilitates water diffusion during cryopreservation. Fertil Steril, 2013, 99: 1428–1435

132 Lucena E, Bernal DP, Lucena C, Rojas A, Moran A, Lucena A. Successful ongoing pregnancies after vitrification of oocytes. Fertil Steril, 2006, 85: 108–111

133 Bhut M, Yaqoob S, Khan F, Waheed S, Sharma V, Vajta G, Ganai NA, Shah RA. Open pulled straw vitrification of in vitro matured sheep oocytes using different cryoprotectants. Small Ruminant Res, 2013, 112: 136–140

134 Cuello C, Sanchez-Osorio J, Almnutna C, Gil M, Perals M, Lucas X, Roca J, Vazquez JM, Martinez EA. Effect of the cryoprotectant concentration on the in vitro embryo development and cell proliferation of OPS-vitrified porcine blastocysts. Cryobiology, 2008, 56: 189–194

135 Chian RC, Kuwayama M, Tan L, Tan J, Kato O, Nagaï T. High survival rate of bovine oocytes matured in vitro following vitrification. J Reprod Dev, 2004, 50: 685–696

136 Chian R, Son W, Huang J, Cui S, Bucket W, Tan S. High survival rates and pregnancies of human oocytes following vitrification: preliminary report. Fertil Steril, 2005, 84: S36

137 Fahy GM, Wowk B, Wu J, Paynter S. Improved vitrification solutions based on the predictability of vitrification solution toxicity. Cryobiology, 2004, 48: 22–35

138 Kasai M, Komı J, Takakamo A, Tsudera H, Sakurai A, Machida T. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. J Reprod Fertil, 1990, 89: 91–97

139 Rho GJ, Lee SL, Kim YS, Yeo HJ, Ock SA, Balasubramanian S, Choe SY. Intracytoplasmic sperm injection of frozen-thawed bovine oocytes and subsequent embryo development. Mol Reprod Dev, 2004, 68: 449–455

140 Carroll J, Wood MJ, Whittingham DG. Normal fertilization and development of frozen-thawed mouse oocytes: protective action of certain macromolecules. Biol Reprod, 1993, 48: 606–612

141 Nagashima H, Nottle M, Kashiwazaki N. Cryopreservation of porcine embryos. Geneva, Switzerland WIPO Patent, 1995005075, 1995-02-24

142 Men H, Agca Y, Riley LK, Critser JK. Improved survival of vitrified porcine embryos after partial delipation through chemically stimulated lipolysis and inhibition of apoptosis. Theriogenology, 2006, 66: 2008–2016

143 Moazad AR, Tan SL, Xu B, Chen HY, Taketo T. L-carnitine supplementation during vitrification of mouse oocytes at the germlinal vesicle stage improves preimplantation development following maturation and fertilization in vitro. Biol Reprod, 2013, 88: 1–8

144 Takahashi T, Inaba Y, Somfai T, Kanaeda M, Geshi M, Nagai T, Manabe N. Supplementation of culture medium with L-carnitine improves development and cryotolerance of bovine embryos produced in vitro. Reprod Fert Develop, 2013, 25: 589–599

145 Chankitisakul V, Somfai T, Inaba Y, Techakumphu M, Nagai T. Supplementation of maturation medium with L-carnitine improves cryo-tolerance of bovine in vitro matured oocytes. Theriogenology, 2013, 79: 590–598

146 Cobo A, Kuwayama M, Pérez S, Ruiz A, Pellicer A, Rennó H. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the Cryotop method. Fertil Steril, 2008, 89: 1657–1664

147 Prentice-Biensch JR, Singh J, Mapletofi RJ, Anzar M. Vitrification of immature bovine cumulus-oocyte complexes: effects of cryoprotectants, the vitrification procedure and warming time on cleavage and embryo development. Reprod Biol Endocrinol, 2012, 10: 73

148 Merlo B, Iacono E, Regazzini M, Zambelli D, Cat blastocysts produced in vitro from oocytes vitrified using the cryo-loop technique and cryopreserved electroacculated semen. Theriogenology, 2008, 70: 126–130

149 Chian RC, Gilbert L, Huang JY, Demirtas E, Holzer H, Benjamin A,
Buckett WM, Tulandi T, Tan SL. Live birth after vitrification of in vitro matured human oocytes. Fertil Steril, 2009, 91: 372–376

150 Mukaida T, Nakamura S, Tomiyama T, Wada S, Oka C, Kasai M, Takahashi K. Vitrification of human blastocysts using cryoloops: clinical outcome of 223 cycles. Hum Reprod, 2003, 18: 384–391

151 Vanderzwalmen P, Bertin G, Debauche C, Standaert V, Bollen N, Van Roosendaal E, Vandervorst M, Schoysman R, Zech N. Vitrification of human blastocysts with the Hemi-Straw carrier: application of assisted hatching after thawing. Hum Reprod, 2003, 18: 1504–1511

152 Cuello C, Berthelot F, Martinat-Botte F, Venturi E, Guillouet P, Vazquez I, Roca I, Martínez EA. Piglets born after non-surgical deep intrauterine transfer of vitrified blastocysts in gilts. Anim Reprod Sci, 2005, 85: 275–286

153 Bielanski A. A review of the risk of contamination of semen and embryos during cryopreservation and measures to limit cross-contamination during banking to prevent disease transmission in ET practices. Theriogenology, 2012, 77: 467–482

154 Desai NN, Goldberg JM, Austin C, Falcone T. The new Rapid-i carrier is an effective system for human embryo vitrification at both the blastocyst and cleavage stage. Reprod Biol Endocrinol, 2013, 11: 41–50

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.