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

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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

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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

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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].

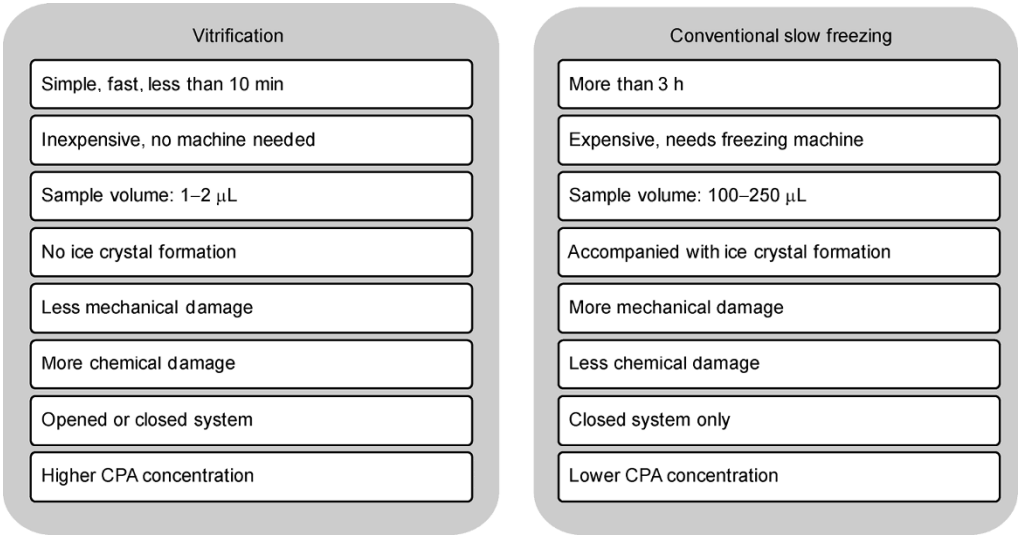

Figure 1 Vitrification versus conventional slow freezing.

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 bisection 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	References
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 oocyte	Cryotop	48.7 vs. 47.5	[146]
Cattle	Immature oocytes	Cryotop	1.6 vs. 34.4	[147]
Cat	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.

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