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Cryopreservation of Oocytes and Embryos: Current Status and Opportunities

Arindam Dhali, Atul P. Kolte, Ashish Mishra, Sudhir C. Roy and Raghavendra Bhatta

Abstract

The biochemical and metabolic activities of living cells are virtually stopped at ultralow temperature and they enter into a suspended state of animation. However, as such, exposure of living cells to ultralow temperature is associated with complex changes that reduce their survivability following freeze-thawing. Cryopreservation is the method for preserving living cells at ultralow temperature at genetically and physiologically stabilized state. Cryopreservation of oocytes and embryos is an integral part of the assisted reproductive technologies with many potential applications. Cryobanking of oocytes and embryos derived from genetically superior animals is promising for enhancing the outcome of planned breeding programs and conserving biodiversity of endangered animal species. Cryobanking can also ensure steady supply of oocytes and embryos for many downstream applications of assisted reproduction such as in vitro embryo production, embryo transfer, production of stem cells, and genetic engineering. Tremendous advancements have been made in this field over the past 5 decades and several methods have been demonstrated for cryopreserving oocytes and embryos in different species. This chapter focuses on the fundamental aspects of oocyte and embryo cryopreservation.

Keywords: cryopreservation, oocyte, embryo, methods, challenges

1. Introduction

The extent of metabolic processes and cellular functions of living cells are reduced dramatically in response to low temperature [1]. Further, at ultralow temperature, the biochemical and metabolic activities of living cells are virtually stopped and they enter into a suspended state of animation. Nevertheless, the exposure of living cells to ultralow temperature induces complex changes to the cells that are associated with its altered physical structure and biophysical processes. These facts indicate that living cells can be preserved at ultralow temperature for a long time. However, the preserved cells will be able to resume their normal physiological functions following recovery, if their physical structure and vitality are protected during the process and period of preservation. The methods for preserving living cells at ultralow temperature essentially employ these principles and the process is known as cryopreservation. It is the technique for preserving living cells or tissues at ultralow temperature, typically in liquid nitrogen (-196°C), at genetically and physiologically stabilized state.

Species	Oocyte	Embryo
Cow	1992 [3]	1973 [4]
Rabbit	1989 [5, 6]	1974 [7]
Sheep	—	1976 [8]
Goat	—	1976 [9]
Horse	2002 [10]	1982 [11]
Pig	2014 [12]	1989 [13]
Buffalo	—	1993 [14]

Table 1.
First reported birth from the cryopreserved oocytes and embryos in different livestock species.

Preservation of oocytes and embryos is an integral part of the assisted reproductive technologies. It allows not only preserving the valuable female germplasm but also the rapid induction of genetic merits into population through in vitro fertilization and embryo transfer. The mammalian embryos could be cryopreserved successfully for the first time in 1972. It was shown that 50–70% of the early stage mouse embryos survived freezing to -196°C that required slow cooling and slow warming [2]. Subsequently, considerable efforts have been made until now to cryopreserve oocytes and embryos in different mammalian species including livestock (**Table 1**). The field of gamete cryobiology has undergone a tremendous advancement during the last five decades.

Cryopreservation of mammalian gamete has many potential applications. Cryobanking of oocytes and embryos derived from genetically superior animals is promising for enhancing the outcome of planned breeding programs. The technique is equally important for conserving biodiversity of endangered animal species and, valuable and genetically modified laboratory animals. It also ensures steady supply of oocytes and embryos for many downstream applications of assisted reproduction such as in vitro embryo production, embryo transfer, production of stem cells, and genetic engineering.

The underlying effects of cryopreservation on mammalian oocytes and embryos have been studied extensively by the scholars worldwide. Several methods have been demonstrated for cryopreserving oocytes and embryos in different species and some of these methods are real breakthroughs. Currently, devices and consumables required for cryopreservation are available commercially from many firms that transform the procedure into a routine practice in humans as well as in livestock. In this chapter, the fundamental aspects of oocyte and embryo cryopreservation are discussed in detail.

2. Principles of cryopreservation

The process of cryopreservation exposes the cells to very low temperatures for preserving their structural and functional entity for a long period of time. As such, the freezing of cells results in the formation of both intracellular and extracellular sharp ice crystals that damage the cellular membranes and organelles and render the cells nonviable. Further, the formation of ice crystals causes osmotic stress to the cells that result from the altered concentration of intracellular solutes. Therefore, any cryopreservation protocol fundamentally includes steps that prevent and ameliorate such damages to cells during freezing. These damages are avoided by controlling the temperature during the freezing process and by incubating cells to cryoprotective solution [15]. A rapid freezing process helps avoiding the mechanical damage caused by the piercing action of ice crystals, and the rise in intracellular solute concentration

can be avoided by exposing cells to cryoprotectants [15]. Permeating cryoprotective agent (CPA) decreases ice formation by replacing the intracellular liquid [16]. Irrespective of the methods, cryopreservation of oocytes and embryos basically includes four steps (**Figure 1**). Step 1: cells are equilibrated in a CPA solution that causes water egress from the cells and their dehydration. Replacement of intracellular water with permeable CPA lowers the freezing point of the intracellular content and reduces the extent of intracellular ice crystal formation. Step 2: equilibrated cells are cooled to low temperature and then stored in liquid nitrogen (-196°C). The cells are actually frozen during this step and, depending upon the cooling methods, either small intracellular ice crystals are formed (slow cooling) or the intracellular content is transformed into glass-like state bypassing ice crystal formation (vitrification). Step 3: cryopreserved cells are recovered by thawing and warming that reverse the frozen state of the cells. Step 4: finally, the thawed and warmed cells are

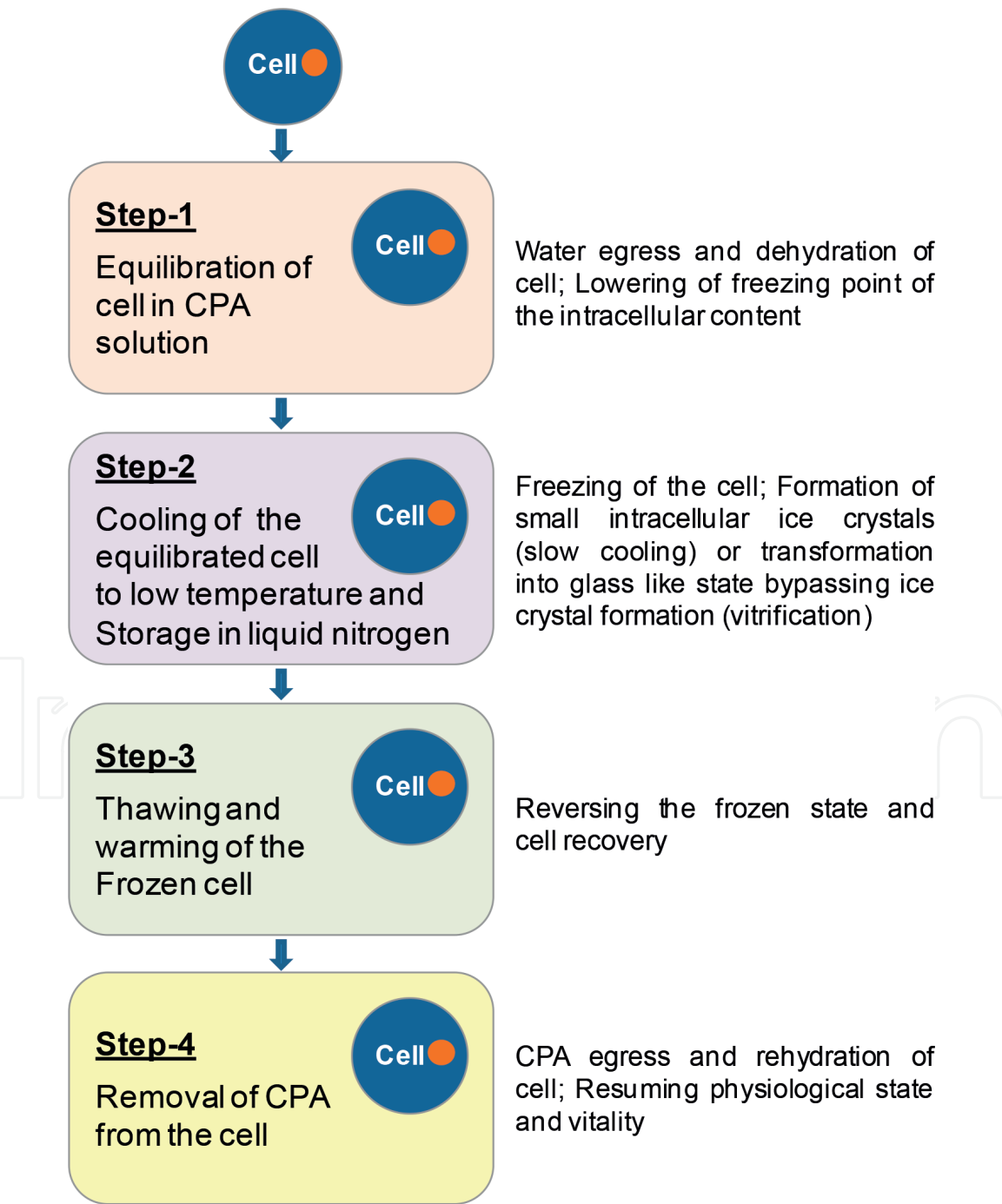


Figure 1.
Steps involved in cryopreservation of oocytes and embryos.

equilibrated in the rehydration solution that causes the replacement of intracellular CPA with water molecules. Following this step, the preserved cells regain their vitality and resume normal physiological processes.

3. Cryoprotectants

CPAs play key roles in protecting the vitality of the cryopreserved cells during their processing and storage at ultralow temperature and their subsequent recovery with normal physiological functionality. Christopher Polge and his colleagues discovered the cryoprotective capabilities of glycerol in the late 1940s that subsequently led to the successful cryopreservation of cattle and poultry spermatozoa [17]. This discovery introduced a fascinating branch of bio-physical science, the cryobiology. CPAs are water-soluble chemical substances with low level of cytotoxicity that lower the melting point of water. CPAs can be divided into two categories, membrane permeating and membrane non-permeating.

Membrane-permeating CPAs are small molecules that easily penetrate the cell membranes. During cryopreservation, these agents decrease freezing point and prevent cell damage from high electrolyte concentrations. They form linkages with the electrolyte molecules and thus act as partial substitute to water [18]. Penetrating CPAs also stabilize lipid membranes by hydrogen bonding with the membrane lipids, which is especially important under severely dehydrated conditions.

On the other hand, the non-permeating CPAs increase the viscosity of the cryopreservation solution generating osmotic gradient across the cell membrane and thus withdraw intracellular water [19]. These agents allow effective dehydration of the cells even in the presence of permeating CPAs at low concentration. Additionally, non-permeating CPAs also reduce mechanical stress that occurs during cryopreservation [20]. The list of CPAs commonly used for cryopreservation of oocytes and embryos is provided in **Table 2**.

It may be noted that no permeating CPA is completely devoid of the capability to induce cell toxicity. Therefore, the use of a single permeating CPA substantially increases the possibility of cellular toxicity to the frozen cells, because of their high concentration in the cryopreservation solution. In contrast, similar viscosity of cryopreservation solution can be achieved by using a combination of permeating CPAs along with non-permeating cryoprotective agents. In the latter case, the

Permeating cryoprotectants	Non-permeating cryoprotectants
Ethylene glycol	Glucose
Dimethyl sulfoxide	Sucrose
Propylene glycol	Trehalose
Formamide	Raffinose
Acetamide	Polyvinyl pyrrolidone
Dimethyl acetamide	Mannitol
	Ficoll
	Polyethylene glycol
	Polyvinyl alcohol
	Bovine serum albumin

Table 2.
Commonly used cryoprotectants for cryopreservation of oocytes and embryos.

concentration of a particular permeating CPA would be much lower in the solution. Therefore, it is believed that the CPA-mediated toxicity to the cells can be reduced if a combination of CPAs is used for cryopreservation.

4. Methods of cryopreservation

Fundamentally, two methods are available for cryopreserving oocytes and embryos. These are slow freezing and vitrification. The major differences between these two methods are the concentration of CPA used and the rate of cooling of sample during the preservation process (Table 3). However, an improved and modified version of the traditional vitrification process has been developed later that utilizes extremely high cooling rate as compared to the slow freezing or traditional vitrification procedures (Table 3). This improved vitrification method is known as ultrarapid vitrification.

4.1 Slow freezing

The technique of slow freezing was introduced first for cryopreserving oocytes and embryos. The technique was developed during the early 1970s [2, 21, 22] and it was considered as a gold standard for long for cryopreserving oocytes and embryos. In this technique, samples are equilibrated to a concentration gradient of

Particulars	Methods		
	Slow freezing	Conventional vitrification	Ultrarapid vitrification
Concentration of cryoprotectants	Low, 1–2 M	High, 5–7 M	Moderate, 3.5–5.5 M
Sample volume	>100 µl	50–100 µl	≤5 µl
Cooling procedure	Slow and controlled cooling with the help of a programmable freezing machine	No freezing machine is required; samples are directly plunged into liquid nitrogen	No freezing machine is required; samples are directly plunged into liquid nitrogen or placed onto a surface cooled to the temperature of liquid nitrogen
Sample processing time	Extended, 2–3 h	Short, <10 min	Short, <10 min
Formation of ice crystal	Yes	No	No
Likely osmotic injury	Low	High	Moderate
Likely CPA-mediated toxic injury	Low	High	Moderate
Likely chilling injury	High	Low	Low
Sample container	Conventional 0.25-ml straw	Conventional 0.25-ml straw	Container less or specialized container other than conventional straw
Status of system	Closed	Closed	Closed or open
Requirement of skill	Easy to perform	Difficult to perform	Difficult to perform

Table 3.
Comparison among the methods of slow freezing, vitrification, and ultrarapid vitrification.

CPAs (1–2 M final concentration) to minimize chemical and osmotic toxicity and to maintain a balance between the factors that influence cell damage [23]. Following equilibration, the samples are loaded into straws and cooled at 1–2°C/min to –5 to –7°C and then seeded to initiate extracellular freezing. Thereafter, the samples are cooled slowly at 0.3–1°C/min until they attain the temperature anywhere between –30 and –70°C [24, 25] and finally the samples are plunged into liquid nitrogen for storage. The controlled cooling of samples is achieved with the help of a programmable freezing machine. During controlled cooling, exchange of water molecules takes place between the extracellular and intracellular fluids without adverse osmotic effects [26]. Nevertheless, the extracellular and intracellular water precipitate and form ice crystals during slow cooling [27].

4.2 Conventional vitrification

Vitrification is an alternative to the slow freezing technique. This method allows solidification of the cell and the extracellular milieu into a glass-like state bypassing the formation of ice crystals. The first successful event of vitrification was reported in 1985 [16], and ice-free cryopreservation of mouse embryos at –196°C was demonstrated. Thereafter, enormous efforts have been made worldwide to utilize and improve this technique for cryopreserving oocytes and embryos in different species. Vitrification is now considered to be a proven method of cryopreservation. In this technique, cells are incubated in CPA solutions from low to high viscosity, loaded into straw, and directly plunged into liquid nitrogen. The process requires much greater cooling rate during freezing and high concentrations of CPA as compared to slow freezing. There are three important factors that ensure the vitrification process:

Cryopreservation technique	Cooling rate	Warming rate
Slow freezing	1–2°C/min until –5 to –7°C followed by 0.3–1°C/min until –30 to –70°C and then plunging sample into liquid nitrogen	250–600°C/min
Conventional vitrification	2000–2500°C/min	250–600°C/min
Ultrarapid vitrification		
Open pulled straw	20,000°C/min	20,000°C/min
Closed pulled straw	8100°C/min	—
Electron microscopic grid	180,000°C/min	—
Cryoloop	15,000°C/min	45,000°C/min
Hemi-straw	>20,000°C/min	—
Cryotop	23,000°C/min	42,000°C/min
CryoTip	12,000°C/min	24,000°C/min
Quartz micro-capillary	250,000°C/min	—
Glass capillary	12,000°C/min	62,000°C/min

Table 4.
Cooling and warming rates of different vitrification methods.

(1) viscosity of the CPA solution; (2) cooling rate; and (3) sample volume. Thus, a delicate balance must be maintained among these factors to achieve successful vitrification [1]. Vitrification can be achieved with a CPA concentration of 5–7 M and a cooling rate of approximately 2500°C/min [28]. A major advantage of vitrification is the low risk of freezing injury, thereby ensuring a sufficiently high cell survival rate.

4.3 Ultrarapid vitrification

Ultrarapid vitrification is the modified and improved version of the conventional vitrification procedure. The concept of ultrarapid cooling for cryopreserving oocytes and embryos was introduced by Vajta and his co-workers with their invention of the open pulled straw [29, 30]. The viscosity of the vitrification medium and cooling rate are inversely related. Thus, a medium containing lesser concentration of cryoprotectants and other additives can be vitrified efficiently at higher cooling rate. Theoretically, vitrification can be achieved with a 1.5 M concentration of any cryoprotectant, providing a cooling rate of 15,000°C/min is employed [28]. Ultrarapid vitrification technique employs extremely high cooling and warming rates as compared to the slow freezing or conventional vitrification methods (**Table 4**). It allows vitrification of relatively low concentration of CPA solution using extremely high cooling rate and thus reduces the CPA-mediated toxicity and osmotic stress to the vitrified cells. The ultrarapid cooling rate is achieved by reducing the effective volume of the solution to be vitrified. At present, this method is considered to be the most superior, and high post freeze-thaw survival of oocytes and embryos has been demonstrated using this method in different mammalian species.

5. Cryoinjury

Oocytes and embryos are susceptible to different types of injuries following cryopreservation, which are collectively known as cryoinjuries. The extent of cryoinjuries to the frozen oocytes and embryos depends on many factors. The major factors are the type of CPA and freezing technique used for cryopreservation and the physiological quality, chilling sensitivity, plasma membrane permeability, tolerance for osmotic stress, developmental stage, and species of the oocytes and embryos [26, 31]. One of the primary focuses during the development of cryopreservation protocols is to minimize the possibilities of cryoinjuries to the preserved oocytes and embryos following freeze-thawing. The different types of cryoinjuries that oocyte and embryos are exposed to during the cryopreservation process are described below.

5.1 Chilling injury

Chilling injuries refer to the irreversible changes that occur to the intracellular lipid droplets, lipid-containing membranes, and the cytoskeleton, during the cooling phase between +15 and –5°C [32]. Such injuries are commonly associated with the slow freezing technique. In contrast, the vitrification method substantially reduces the chances of chilling injuries to the frozen oocytes and embryos as they are exposed very briefly to the dangerous temperature zone due to high cooling rate [33]. Therefore, effective cryopreservation of porcine embryos containing extremely large amounts of chill-sensitive lipid droplets can be achieved only through vitrification [34]. Similarly, high survivability of cryopreserved oocytes of various other species such as cattle, sheep, and horse that are sensitive to chilling could be achieved through vitrification [35].

5.2 Formation of ice crystal

The formation of ice crystals during cryopreservation is the major source of cryoinjury [36]. The slow freezing method induces ice crystal formation in the aqueous phase surrounding cells as well as inside the cells including the cytoplasm and nucleus at the temperature zone between -5 and -80°C . In contrast, high CPA concentration and rapid cooling rate of vitrification method allow solidification of intracellular and extracellular water into a glass-like state bypassing the formation of ice crystals.

5.3 Fracture damage

Fracture damages to the zona pellucida and blastomeres of oocytes and embryos are commonly observed following cryopreservation. Such damages usually occur during freezing because of the mechanical effect of the solidified solution at the temperature zone between -50 and -150°C [37].

5.4 Formation of multiple asters

Aster formation is a newly discovered form of cryoinjury. It is frequently observed in the vitrified-warmed and fertilized oocytes [38]. This cryoinjury is likely accountable for the loss of ooplasmic function responsible for normal microtubule assembly. The exposure of oocytes to high CPA concentration and ultrarapid cooling during vitrification leads to the formation of multiple asters near the male pronucleus. The migration and development of pronuclei are disrupted by the asters resulting in delayed first cleavage and reduced blastocyst development [38].

5.5 Osmotic stress

During the pre-freezing stage of cryopreservation, incubation of cells with high osmolar cryoprotectant solution causes cell shrinkage due to the outward movement of intracellular water in response to the difference in osmotic pressure between intracellular and extracellular solutions. Similarly, at the stage of thawing and CPA removal, the movement of water molecules occurs at the reverse direction that causes cell swelling. These phenomena are known as osmotic stress. The frozen cells are more permeable to water than cryoprotectants as compared to their fresh counterpart [39]. Therefore, the cryopreserved cells are more susceptible to osmotic stress as compared to the non-cryopreserved cells. The vitrification method employs considerably high concentration of CPAs and therefore induces greater osmotic stress as compared to the slow freezing technique. It may be noted that the required CPA concentration for vitrification is inversely related with the cooling rate. Therefore, a practical approach to reduce osmotic stress and CPA-mediated cell toxicity during vitrification is to increase the cooling rate and simultaneously reduce the concentration of CPAs.

6. Deleterious effects of cryopreservation

Cryopreservation of oocytes and embryos is associated with several deleterious consequences that in turn exert negative effects on their post freeze-thaw survivability and development.

Osmotic shock during cryopreservation and thawing may result in excessive shrinkage or swelling of cells that can damage the cellular cytoskeleton and in turn

the post freeze-thaw survivability and developmental ability of the cryopreserved cells. Similarly, the formation of intracellular ice crystals during freezing may damage the cellular cytoskeleton and cell organelles.

Mitochondria are the most abundant organelles in mammalian oocytes and embryos and they are the sole source of energy production. Mitochondrial dysfunction or abnormalities are critical for the development of oocytes and embryos. A reduction in the production of ATP by mitochondria is associated with the developmental failure of oocytes and embryos [40]. Cryopreservation may contribute to mitochondrial dysfunction, mitochondrial swelling [41, 42], abnormally shaped mitochondria, rupture of mitochondrial membranes [43, 44], and reduced cellular ATP content that might contribute to poor oocyte and embryo development following freeze-thawing [45, 46].

It is evident that cryopreservation incurs negative effect on the expression of genes associated with oxidative stress, apoptosis, cell developmental process, and sperm-oocyte interaction [31, 47]. Such alteration in gene expression is one of the contributory factors of cryopreservation toward poor developmental ability of cryopreserved oocytes and embryos.

Cryopreservation can be a potential cause of physical damage to DNA. The fragmentation of DNA increases in mouse and bovine oocytes following vitrification [48, 49]. It is suggested that slow freezing as well as vitrification affect the DNA integrity in embryos [50]. Further, cryoprotectants such as ethylene glycol and propanediol increase DNA fragmentation in porcine embryos, even without a cycle of freezing and thawing [51].

Cryopreservation may induce epigenetic changes in the genome of cryopreserved oocyte and embryos. Vitrification reduces or increases gene methylation in bovine and mouse oocytes and embryos [52–55]. Further, several reports indicate that vitrification significantly alters acetylation patterns in oocytes [56, 57]. It is suggested that the aberrant epigenetic modifications in response to cryopreservation are at least partially responsible for the reduced developmental competence of frozen oocytes and embryos [31].

7. Difficulties associated with oocyte cryopreservation

The cryopreservation of oocyte is more challenging than that of the embryos. As compared to an embryo, an oocyte has to maintain integrity of many of its unique structural features following freeze-thawing to undergo fertilization and further development. Oocyte being a single cell is more vulnerable to the steps of cryopreservation as compared to a multi-cellular preimplantation embryo. The larger volume of oocyte decreases the surface-to-volume ratio that makes it very sensitive to chilling and intracellular ice formation [58, 59]. The plasma membrane of matured oocytes has a low permeability coefficient, thus making the movement of cryoprotectants and water slower [60].

In oocytes, the meiotic spindles play crucial roles in meiotic progression as well as chromosomal alignment and segregation [61]. Severe disorganization or disappearance of meiotic spindles is evident following slow freezing as well as vitrification with a more deleterious effect of the slow freezing procedure [31]. Cryopreservation exerts a negative influence on microfilament functions in oocytes that in turn can lead to abnormal distributions of mitochondria in the oolemma [6, 62, 63]. This consequently may result in reduced meiotic competence and fertilization ability of oocytes and developmental failure of early stage embryos.

During cryopreservation, CPA causes transient increase in the intracellular concentration of calcium in oocytes [64] that triggers exocytosis of cortical

granule [65] resulting in hardening of zona pellucida and in turn compromised sperm penetration and fertilization [66].

8. Future perspectives

The procedures of oocyte and embryo cryopreservation have evolved significantly since it was demonstrated for the first time five decades ago. Nevertheless, the success of oocyte cryopreservation is considerably poor as compared to that of the embryos at late developmental stage, even following the ultrarapid vitrification, which is considered as the best technique at present. Therefore, currently, the most important challenge in this field is to develop standardized protocols for effective cryopreservation of oocytes and early stage embryos. The theoretical target of success of such protocols should be comparable with that of their non-cryopreserved counterpart. It is evident from the current state of knowledge that the ability of oocytes and embryos to withstand cryopreservation process varies among the different species. It appears impossible to develop a single standardized protocol for all species. Therefore, future efforts should focus on developing species-specific optimized protocols for oocyte and embryo cryopreservation. Further, it will be fascinating to observe future efforts for the development of automated devices for oocyte and embryo vitrification. The implementation of an efficient and automated ultrarapid vitrification system for routine use in livestock can revolutionize the field worldwide. Conclusively, the most prominent future targets of cryopreservation are expected to focus on the development of protocols that would maintain as much as possible the structural and functional integrities of oocytes and embryos following freeze-thawing. The outcome of such protocols should be reproducible as well across the laboratories worldwide. Realization of such targets would definitely lead to the development of standardized and optimized methods for oocyte and embryo cryopreservation for routine use in livestock.

9. Conclusions

Cryopreservation is the technique to preserve living cells at ultralow temperature, typically in liquid nitrogen (-196°C). Cryopreservation of oocytes and embryos is extremely important for propagation and conservation of genetically superior germplasm. Any cryopreservation protocol basically includes three major steps such as equilibration of cells to concentrated solution of cryoprotective agent, cooling and storage of cells to ultralow temperature, and recovery of frozen cells following thawing and warming. Cryoprotective agents protect vitality of the cryopreserved cells during processing and storage at ultralow temperature. Basically, there are two fundamental methods for cryopreserving oocytes and embryos, slow freezing and vitrification. The slow freezing method involves cooling of the samples slowly at controlled rate and formation of intracellular and extracellular ice crystals. In contrast, the conventional vitrification method involves rapid cooling of the samples and solidification of the cells including extracellular milieu into a glass-like state bypassing ice crystal formation. Ultrarapid vitrification is the modified and improved version of the conventional vitrification procedure that involves extremely high cooling and warming rates. Currently, ultrarapid vitrification is considered to be the more superior method than slow freezing or conventional vitrification. It is evident that cryopreservation often results in different types of cryoinjuries such as chilling injury, formation of ice crystal, fracture damage, osmotic stress, and formation of multiple asters. The quantum of cryoinjuries to

frozen cells depends on many factors. Cryoinjuries are responsible for poor survivability of the cryopreserved cells. The cryopreservation process is associated with several other deleterious consequences and those in turn exert negative effects on the post freeze-thaw survivability of the frozen cells. Cryopreservation of oocytes is more difficult and yields poor success because of their larger volume and unique structural features as compared to that of the embryos. The procedures of oocyte and embryo cryopreservation have evolved significantly over the past five decades. Yet, the species-specific optimized cryopreservation methods with reproducible results are not available currently, especially for the oocytes and early stage embryos.

Conflict of interest


None.

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