Chapter

Cryopreservation Methods and Frontiers in the Art of Freezing Life in Animal Models

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Abstract

The development in cryobiology in animal breeding had revolutionized the field of reproductive medicine. The main objective to preserve animal germplasm stems from variety of reasons such as conservation of endangered animal species, animal diversity, and an increased demand of animal models and/or genetically modified animals for research involving animal and human diseases. Cryopreservation has emerged as promising technique for fertility preservation and assisted reproduction techniques (ART) for production of animal breeds and genetically engineered animal species for research. Slow rate freezing and rapid freezing/vitrification are the two main methods of cryopreservation. Slow freezing is characterized by the phase transition (liquid turning into solid) when reducing the temperature below freezing point. Vitrification, on the other hand, is a phenomenon in which liquid solidifies without the formation of ice crystals, thus the process is referred to as a glass transition or ice-free cryopreservation. The vitrification protocol applies high concentrations of cryoprotective agents (CPA) used to avoid cryoinjury. This chapter provides a brief overview of fundamentals of cryopreservation and established methods adopted in cryopreservation. Strategies involved in cryopreserving germ cells (sperm and egg freezing) are included in this chapter. Last section describes the frontiers and advancement of cryopreservation in some of the important animal models like rodents (mouse and rats) and in few large animals (sheep, cow etc).

Keywords: cryopreservation, fertility, reproduction, cryobiology

1. Introduction

Ever since the human evolution and civilization, human has been exploiting animals either for food, transport and or as companions. The use of animals in biomedical and behavioral research has greatly increased scientific knowledge and has benefitted human health enormously. Tremendous advancement took place in the field of medical sciences with the usability of animals for experimental research. Currently, around 75–100 million vertebrates are used annually in research and testing [1]. The most frequently used animals are mice and rats that constitute approximately 95% of experimental animals; mouse being the most commonly used animal in biomedical research [1]. Animal models are used in research for wider

understanding of vital physiological processes in human and animals. Animal models are also useful in investigating various diseases including metabolic disorders such as diabetes, cardiovascular disease (CVD), disorders in reproductive endocrinology, infertility, cancer and infectious diseases [2–7]. Human lives endangered due to organ failure were restored after successful organ transplantation accomplished in animal models. Optimization of cryopreservation protocols will significantly facilitate organ transplantation and/or replacement. In addition, animals like dogs, pigs, cats, sheep, non-human primates (NHP) and fish are widely used for genetic and physiological studies in human health and disease [8]. The chapter is focused on the fundamentals of cryobiology and strategies in male (sperm) and female fertility (oocyte and ovarian tissue) cryopreservation. The last section is focused on the frontiers in cryopreservation of most widely used animal models like rodents and higher animals used in biomedical research and toxicological studies.

1.1 Necessity to cryopreserve animal's genetic resources?

Large numbers of animal breeds worldwide are either extinct or endangered with few at the verge of extinction. Hence, it is crucial to develop and apply rescue strategies to ensure survival of these species for the future. One way is to preserve the genetic resources or the germplasm of these species for their maintenance and future development. Genetic diversity is another threat resulting from animal husbandry errors that can result in genetic drift of existing colonies and genetic contamination of lines. Furthermore, weather related natural disaster is a major threat to animal husbandry and vivarium, especially in case of experimental animals that require special care in breeding and rearing colonies. Cattle and breeding industries are modified for the large-scale production of the animal species. There is an urgent need to improve the efficiency and sustainability of producing animals for food in the face of the ever-increasing world population. Improved understanding of mechanisms and challenges of reproductive technologies are vital for improving the viability of the livestock industry [9]. Hence, one solution to preserve animal species is by freezing. Cryopreservation has emerged as the most efficient and compatible method for freezing human and animal genetic resources [10]. Cryobiology is an integrated study of various biological and physical sciences. Semen, embryos, oocytes, somatic cells, nuclear DNA, and other types of biological material such as blood, serum, and tissue, can be stored using cryopreservation, in order to preserve genetic materials or for other applications [8, 11]. The primary benefit of cryopreservation is the ability to save germplasms for extended periods of time, and thus maintaining the genetic diversity of a species or a breed [12]. Also, germplasm of genetically engineered animals (GEA) and cell lines of various species can be preserved by cryopreservation method. Gene banks/cryobanks are established and contain repository of cryopreserved genetic resources to regenerate a particular population in the future [13–15]. Sperm cryopreservation has been successfully applied in various fields to benefit the mankind and animals. Of prime significance, assisted reproduction technology (ART), the forefront in infertility treatment today might be inconceivable without the efficient cryopreservation techniques.

1.2 History behind the discovery

Spallanzani's observation in the beginning of eighteenth century snow cooling the sperm was a breakthrough in the field of biology. Sperm was found to maintain

motility and viability even when exposed to cold temperature conditions [16]. Later, in the nineteenth century a major breakthrough occurred with the successful cattle insemination performed using cryopreserved samples. A successful protocol for sperm freezing and storage at low temperatures (−79°C) was developed by Polge *et al.,* [17]. Spermatozoon was the first mammalian cell to be successfully frozen [18]. This significant success in sperm freezing was associated with the discovery of glycerol as a cryoprotectant by Polge *et al* in 1949 [17]. Since 1970 and till date, cryopreservation would not be successful preservation procedure in reproductive medicine without the discovery of plastic straws and cryotubes that tolerate extreme low temperature used in packaging sperm. Mouse embryo freezing was reported using dimethylsulfoxide (DMSO) as a cryoprotectant in 1972 after successfully cryopreserving spermatozoa [19].

2. Art of freezing life

2.1 Cryopreservation methods

Method of cryopreservation and recovery involves following steps. Initially, cells are treated with cryoprotective agents (CPAs) to prevent cryoinjury/damage. Later, cells are cooled in a controlled manner at subzero temperatures, at which the metabolic processes of the cell stops. The recovery of the cells follows a reversed procedure; cells are rapidly thawed and then the CPAs are gradually removed. The viability of the cryopreserved sample is enhanced with the use of appropriate CPA type and concentration and the appropriate rate of freezing [10].

The two basic methods of cryopreservation include the conventional slow freezing (SF) and the rapid freezing method, known as vitrification (Vit). These protocols require different concentrations of CPAs and apply different cooling rates. **Figure 1** illustrates the two methods of cryopreservation.

2.1.1 Slow freezing

Slow freezing involves progressive cooling of sample over a period of 2–4 h either manually or automatically using a semi programmable freezer. This method was developed by Behrman and Sawada [20]. In SF, a phase transition occurs from liquid to solid on temperatures below freezing point. Slow-cooling protocols involve the use of <1.0 M of cryoprotective agents (CPAs), such as glycerol or dimethyl sulphoxide (DMSO), which have minimal toxicity at lower temperatures with the use of a high-cost controlled rate freezer or a benchtop portable freezing container [21].

Main advantage of SF is the reduced risk of contamination during the procedure, without the need of highly skilled professionals. However, SF has many disadvantages. It is time consuming and expensive. There is a high risk of cryoinjury due to the formation of extracellular ice. Although, slow cooling considered a successful method, the success rate is considerably low and might not be suitable for all kinds of cells and tissues [22]. SF is a commonly used method for preservation of animal germplasm for majority of farm animals like sheep, cow, zebrafish etc. [23, 24]. Though with certain drawbacks, nonetheless SF was found more efficient method to cryopreserve ovine embryos in comparison to vitrification [25].

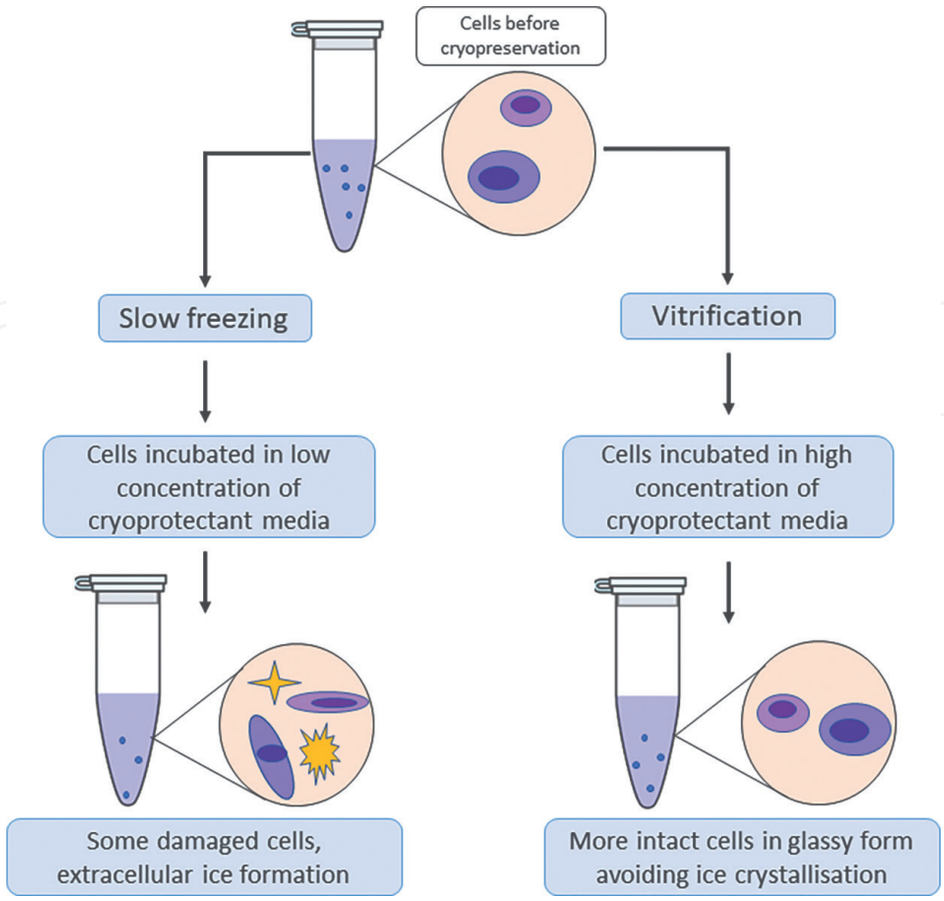

Figure 1.
Comparison of slow freezing and vitrification method.

2.1.2 Vitrification or ice-free cryopreservation

To circumvent the process of ice crystallization, cost and duration for cryopreservation in SF, an alternative technique called rapid freezing or vitrification (Vit) was developed. Vit is an ultrarapid cooling method with high cooling rate, which enables putting cells at cryogenic temperatures, forming what is known as ice-front status, while avoiding ice crystallization, hence also termed ice-free cryopreservation. Vitrification is now the recommended protocol for embryos and cells freezing. The process includes cooling the cells or tissue to cryogenic temperatures using liquid nitrogen, after their exposure to high concentrations of CPAs, with subsequent rapid cooling to avoid ice nucleation [11]. The cell suspensions are transformed directly from the aqueous phase to a glass state upon exposure to LN_2. High CPA concentration and higher cooling and warming rates eliminates ice formation [26]. Several factors like viscosity or thickness of the sample, cooling and warming rates, sample volume, CPA type and concentration have considerable effect on the process. Thus, a balance of all the important factors is required for successful vitrification. Vitrification is of two types—equilibrium and nonequilibrium. Formulation of multimolar CPA mixtures and their injection into the cell suspensions is termed as Equilibrium vitrification. Non-equilibrium vitrification includes the use of carriers like plastic straws or cryoloops for obtaining minimum drop volume and carrier-free systems that employs higher freezing rate and lower CPA mix concentration. In comparison to slow freezing, vitrification is more advantageous as is associated with decreased risk of cryoinjury, thereby ensuring sufficiently higher cell survival rate [11]. To note, there is no

universal protocol followed for the animal species. However, germ cells from species like mouse ovary [27], fish embryos [28], ovarian sheep [29] and many other species has been cryopreserved by vitrification [24].

2.2 Other emerging cryopreservation techniques

2.2.1 Controlled vitrification by liquidus tracking (LT)

Liquidus tracking (LT) is a slow and controlled vitrification protocol with gradual increase in the cryoprotectant concentration simultaneously with continuous decrease of the temperature at subzero ranges in a specified rate. With LT, recovery and restoration of chondrocytes was achieved successfully from cryopreserved articular cartilage [30] and promising results has been reported in case of ovarian tissue cryopreservation [31]. The principle of LT is the dynamic control of CPA concentrations, throughout the cooling process, in order to maintain the cell just above its freezing point at all times, without the formation of ice [11]. An example, Ovarian Tissue Cryopreservation (OTC) by LT has been reported successful in restoration of ovarian function in sheep model [29].

2.2.2 Laser pulse vitrification

Fowler and Toner proposed the applicability of laser light in cryopreservation process. A successful recovery of rapidly frozen red blood cells by vitrification was achieved without the use of CPAs. Principally, laser targets only the intracellular ice causing it to melt and resolidify into glassy state. After thawing and use of laser light, around 80% of cells treated remained viable [32]. In an attempt, Jin *et al.,* [33] reported full survivals of mouse oocytes after vitrification in 3-fold diluted media and ultra-rapid warming by an IR laser pulse [33].

2.2.3 Isochoric and hyperbaric cryopreservation

New approach in the cryopreservation method is freezing under pressure. Previous method discussed above employed constant-standard pressure (isobaric) conditions near 1 atm of pressure. Isochoric (constant-volume) cooling provides means to significantly lower nonfrozen storage temperatures without any or with only minimum requirements for CPAs, achieving greater metabolic reduction without injury associated with freezing, CPA toxicity, or increased amounts of osmotic solutes. The isochoric cryopreservation is a two-phase equilibrium process, in which ice and liquid exist simultaneously at equilibrium under constant temperature and volume, while hyperbaric cryopreservation the solution is maintained in a single phase as liquid, the survival rate is however low in this method [34]. RBCs were successfully cryopreserved by this method [35]. Despite multiple attempts, scientists have not been able to cryopreserve and restore normal functions of complex bio-samples, such as mammalian tissues and organs.

2.3 Fundamentals of cryopreservation and cryoinjury

Prior to the understanding of the role of cryoprotective agents (CPAs), the impact of subzero temperatures on healthy tissues and basic principles in cryobiology must be knowledged. As known, water is one of the most essential elements present in

every cell, tissue, and organ of the living organisms on earth. It constitutes around 80% of tissue mass [36]. On lowering temperature, water undergoes phase transition (liquid to solid) and results in ice crystallization. The formation of intracellular ice cause damage to cellular structure and its function consequently leads to cryoinjury. Freezing can cause two types of harmful effects on cells. The formation of ice crystals damages the cell membrane and thus regain of structurally intact cells on thawing would be difficult. Further, ice formation increases the solute concentration leading to osmotic imbalance and cellular damage. To minimize or to mitigate these effects, two protective actions viz. selection of effective cryoprotectant, and appropriate cooling and thawing rates must be undertaken.

2.3.1 Cryoprotective agents (CPAs)

The discovery of CPA and its role in reducing cryoinjury was a significant step in cryopreservation success. Biological acceptability, cell penetration, low toxicity, are some of the properties, a CPA should possess. As mentioned in previous section, best survival rate of cells and tissues depends on the optimization of factors like cooling rate, warming rate, sample volume. CPA concentration is a major factor influencing the success of the cryopreservation [37]. Based on their penetrating capabilities through cell membrane, CPAs are classified into two categories- membrane permeable/permeating and membrane impermeable/non permeating.

2.3.1.1 Permeating CPAs

Permeating CPAs are smaller sized (typically less than 100 daltons), and amphiphilic in nature [38]. Owing to these properties these molecules can penetrate through the cell membrane easily, tend to equilibrate within the cytoplasm and replace the intracellular water in order to avoid excessive dehydration. Henceforth, they protect the cell from intracellular ice formation (IIF) and salt accumulation. Examples of CPAs in this category are: glycerol (the first agent discovered), dimethyl sulfoxide (DMSO), ethylene glycol (EG), and propanediol (propylene glycol) [39]. The protective role of CPAs is due to hydrogen bonding with water molecules, and lowering the freezing point of water. As a result, less water molecules are available to interact with themselves to form critical nucleation sites required for crystal formation [40]. To minimize toxicity, vitrification mixtures are often added in a stepwise fashion at temperatures near 0°C. Addition of permeating agents prevent the formation of ice and permit cell storage at supercool temperatures. Besides vitrifying, few CPAs, example DMSO have properties to enhance the cell permeability in a dose dependent manner. DMSO of about 5% is reported to increase permeability by reducing the thickness of the cell membrane. DMSO at 10% concentration is more effective and commonly used as it induces water pore formation in biological membranes. Intracellular water can thus easily be replaced by CPAs to promote vitrification. At higher, toxic concentrations (40%) however, lipid bilayers begin to disintegrate [41]. Thus selection of appropriate CPA concentration is vital for maintaining structural integrity and viability after freezing.

2.3.1.2 Non-permeating agents

Unlike permeating, non-permeating agents are covalently linked dimers, trimers or polymers with a larger size. They cannot pass through the cell membrane and exert

their protective effect extracellularly. Most commonly used non-penetrating CPAs are polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), raffinose, sucrose, and trehalose [42, 43]. The mode of action of non-permeating agents is similar to permeating agents by controlling osmolarity but works extracellularly and at a lower degree.

List of different permeating and non-permeating cryoprotective agents used in gamete and embryo cryopreservation in different species is listed in **Table 1**.

Cryoprotectant	**Cell type**	**Species**
Permeating CPAs		
Dimethyl sulphoxide	Oocyte, ovarian tissue, embryo, sperm	Mouse, cow, human
Amides and imides		
Formamide	Sperm	Mouse, horse, dog, goose
Acetamide	Oocyte, embryo	Mouse, rat, rabbit, pig
Propionamide	Sperm	Rabbit
Lactamide	Sperm	Rabbit
Butyramide	Sperm	Rabbit
Malonamide	Sperm	Rabbit
Alcohols		
Methanol	Sperm	Horse
Ethylene glycol	Oocyte, embryo, sperm	Mouse, cow, human
Glycerol	Oocyte, embryo, sperm	Mouse, cow, human
Xylitol	Embryo	Rat
Arabitol	Embryo	Rat
Erythritol	Embryo	Rat
Non-permeating CPAs		
Monosaccharides		
Glucose	Sperm	Cat
Galactose	Sperm	Horse
Disaccharides		
Sucrose	Oocyte, embryo, sperm	Mouse, cow, human
Trehalose	Oocyte, embryo, sperm	Mouse, cow, human
Lactose	Sperm	Mouse, cow
Maltose	Sperm	Mouse, rabbit
Polysaccharides		
Raffinose	Oocyte, sperm	Mouse, horse
Dextran	Embryo	Mouse, cat
Macromolecules		
Polyethylene glycol	Oocyte, embryo	Mouse, human, cow
Ficoll	Oocyte, embryo	Mouse, human, cow
Polyvinyl alcohol	Oocyte, embryo	Mouse, sheep, cow
Hyaluronan	Embryo, sperm	Mouse, sheep, cow

Table 1.
List of different permeating and non-permeating cryoprotectants used in assisted reproductive technologies.

Besides these commonly used CPAs, protein like sericin from silkworm and small antifreeze proteins derived from marine teleosts or fishes have also garnered attention as CPAs in cryobiology [11].

2.3.2 Cooling and thawing rates

Choice of appropriate cooling and thawing rates is another vital step for a successful cryopreservation. Mazur [44] has previously demonstrated the significant correlation between cooling rates and survivability of various cells [44]. The cooling rate was directly proportional to intracellular ice formation and inversely proportional to survivability among various cells. **Figure 2** illustrates the survival of several cell types after cryopreservation in relation to the cooling rate. All cells exhibited a characteristic inverted U-shaped curve indicating that the survival rate increases with an increase in cooling rate upto a point after which the survival gradually decreases. Larger cells dehydrate slowly compared to smaller cells. Hence, in the light of this, rates of cooling and thawing should be adjusted. With an exception to larger cells, a cooling rate of approximately 1°C/min is often recommended. Controlled rate freezers that modulate chamber temperatures are used for this purpose. Following cryopreservation, cells are stored for future thawing and appropriate use.

Unlike cooling rate, thawing rate has been given inadequate attention. Nevertheless, it is advisable to warm or thaw cells rapidly to prevent recrystallization of ice [47]. This can be explained thermodynamically as vitrified state is quasi-stable and can change into a lower energy crystal structure on thawing. Currently, to achieve maximum viability it is suggested to transport cryovials on vapor LN_2 and warmed rapidly in a 37°C water bath for 90–120 s [39]. Decrease in viability after post thaw recovery is inevitable, no matter how well the cells were stored and thawed. Care should be taken to remove dead cells timely using density media like Ficol or other methods from the recovered cells to increase the viability of recovered cell prior to use. Density gradients can be utilized to increase viable cell density although this method often involves exposing cells to additional, potentially-harmful centrifugation. Current strategies for identifying cells that remain viable after preservation utilize organic fluorophores, and dyes.

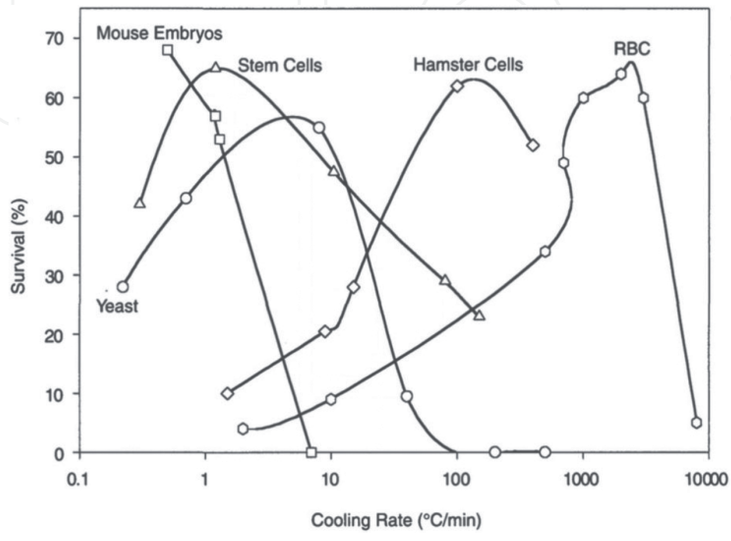

Figure 2.
An inverted U shape curve demonstrating relationship between cooling rate and cell survival (adapted from article by Cipri et al., [45]; figure created originally by Critser and Mobraaten [46] in ILAR Journal).

2.3.3 Recent advances in post-cryo cell recovery

The complete cryopreservation procedure is associated with great efforts to maintain cell viability and function. However, some of the cryopreserved cells often demonstrate decreased viability following thawing. Therefore, it is essential to remove dead cells and increase the viability of live cells. Currently, organic fluorophores are employed for viable cells selection. Viable cell selection using this method is commonly practiced in ART in animal reproduction example semen/spermatozoa of bull, buffalo, rabbit, alpaca, stallion/horse and many other species [48–50]. The earlier practice to achieve this, involved the use of density gradient. Although a little expensive, advanced technologies like magnetic affinity cell separation (MACS), and fluorescence activated cell sorting (FACS) appeared beneficial [51, 52]. Cryosurvival and recovery of sperm in species like stallion, bovine etc. using FACS has been investigated [53, 54]. New strategies like the introduction of nano-science had revolutionized the field of cryobiology and forwarded it to more higher stages as nanoparticle mediated cell sorting is non-destructive and more beneficial than FACS [55].

2.4 Sperm cryopreservation

After the successful live birth with 21 years cryopreserved sperm sample, sperm viability in cryopreserved sample was evident [56]. Currently, sperm cryopreservation as a method of fertility preservation had gained tremendous significance and applicability in human and animals [57, 58]. Sperm survival rate was found to increase with glycerol as CPAs and increasing concentration of glycerol added at constant cooling rate for long term storages [59]. Sperm freezing can be done by one of the main two established methods; slow freezing or ultra-rapid freezing [60, 61]. Owing to the deleterious effects of SF on sperm physiochemical activity and motility, the rapid freezing approach was suggested to be the potential solution to preserve cells without allowing the nucleation of ice crystals. Currently, ultra-rapid freezing adopted ensures both intra and extracellular vitrification thus improving sperm survival [62].

2.5 Female fertility cryopreservation

Like sperm, female germ cells can be frozen by cryopreservation. Fertility preservation in female cancer survivors is a major concern in oncology and assisted conception. Fertility in females can be preserved by freezing egg either embryo, oocytes and oocytes within ovarian tissue. The results with oocyte cryopreservation were unsatisfactory. Ovarian tissue cryopreservation on the other side have attracted the interest of the medical and scientific communities. Cryopreservation protocols for oocyte and ovarian tissue are discussed briefly below.

2.5.1 Oocyte cryopreservation

For oocyte cryopreservation, currently acknowledged methods are SF using equilibrium freezing and Vit/non-equilibrium protocols. In cryopreserving oocytes by SF method, the protocol involves gradual cooling of the specimen to lower temperature (−150°C)with controlled slow cooling rates in presence of low concentration of DMSO (1.5 M) plus non-permeating sugars like sucrose or trehalose at 0.3 M concentration. Specimens are stored at −196°C in LN_2. The survival rate achieved by SF remains relatively unsatisfactory. Evidence from earlier investigations

indicates survival rates plateau around 70–80% with this method [63]. Hence, the recommended method for cryopreservation of oocytes is vitrification. Initially, oocytes are equilibrated with a solution containing PEG and DMSO at 7.5% v/v for 5–15 min. Prior to storing the cells in $LN_{2,}$ the cells are exposed to vitrification media (PEG and DMSO-15% v/v, -and sucrose (0.5 M) for a minute and stored. For thawing, CPAs are gradually removed to avoid ice crystal formation. Later, the cells are revived following incubation in culture medium [64]. Intriguing results were obtained in systematic analysis conducted by Arav and Natan. Vitrified oocytes were reported to have higher oocyte survival and fertility rates compared to slow-cooled oocytes. Furthermore, no differences were observed in pregnancy rate, formation of top quality embryo and fertilization between vitrified and fresh oocytes thus strongly signifying vitrification as the superior procedure for the oocytes cryopreservation [65]. During the past few years, a significant progress has been made in the cryopreservation of oocytes of different species using new vitrification methods. High rates of survival and development after solid-surface vitrification have been reported for *in vitro* matured oocytes from cows [66] and goats [67].

2.5.2 Ovarian tissue cryopreservation (OTC)

Parrot's study, which resulted in first mice offspring was developed following ovarian tissue cryopreservation (at −79°C) and isografting was a breakthrough in the early 1960s [68, 69]. Later, with further discoveries of CPAs like DMSO, propanediol, and ethylene glycol, cryopreservation methods gradually improved. Like oocyte, ovarian tissues are also preserved by SF and Vit method, specially ovarian cortical tissue from mammals [70]. After the advent of Vit for oocyte cryopreservation and in comparison, to SF, ovarian tissue vitrification is now considered promising for ovarian cortical tissue cryopreservation [71, 72]. Ovarian tissue cryopreservation is successfully accomplished in many animal species like mice [68], ewe [73], cow including human [74] and other species.

Table 2 shows the comparison between SF and vitrification protocol for ovarian tissue cryopreservation. Vitrification was suggested to have several advantages over

Features	Slow freezing	Vitrification
Period of freezing process	Slow usually 3–4 h	Fast <10 min
Direct contact with liquid N_2	No	Yes
Ice formation	Yes	No
CPA concentration	Low	High
Cooling rates (°C/min)	0.15–0.3	15,000-30,000
Cost	Expensive	Inexpensive
Special equipment	Yes	No
Techical expertise	Simple	Risky
Sample volume	100–200 μl	1–2 μl
Mechanical damage	More	Less
Chemical change	Less	More

Table 2.
Comparison between SF and vitrification protocol.

the conventional SF in characteristics reviewed. However, SF remains the standard clinical method until further reports show improved success rate for Vit to be applied in human clinical use over SF. Currently, the main purpose for OTC is fertility preservation known so far, especially in young women diagnosed with cancer or some genetic disease that destroys ovarian reserve. Access to immature oocytes from antral follicles and restoration of organ function have evoked new perspectives in utility of OTC for social reasons besides medical use.

2.6 Applications of fertility preservation

Germ cell cryopreservation certainly had emerged as effective method for long term fertility preservation in the field of reproductive medicine in both human and animals. Germ cells cryopreservation would be applicable in restoring fertility in animals and humans and preservation of endangered animal species. Over a decade there has been increase in production of GEA models from cryopreserved animal genetic resources for disease investigations. The practice of genetic engineering has increased the number of mouse and rat lines to tenfold the actual number. Currently, sperm cryopreservation is a fundamental technique in assisted reproduction technology (ART) like artificial insemination (AI), *in vitro* fertilization (IVF), or intra-cytoplasmic sperm injection (ICSI). AI has relatively been the most important practice contributing to the advancement of animal production. Many advantages of AI are enhanced when semen is cryopreserved and stored for extended periods. Several species such as mice, rabbits, pigs, goats, cows, and sheep has been successful reproduced adopting egg and ovarian tissue cryopreservation [66–68, 73, 74]. After successful animal experimentation using cryopreserved germ cells, utility of fertility preservation is gradually extended to human. At present, cryopreservation of human oocytes and sperm has been carried out for medical or social reasons. Infertility in couples is solved to a considerable extend. Cancer patients who are at risk of fertility loss either due to radio or chemotherapy and women who wish to conceive in later ages can benefit from the cryopreservation technology and fertility restoration.

2.7 Frontiers in cryopreservation in animal models

The birth of mouse from 50 years old cryopreserved embryo had revolutionized the field of animal reproduction. Later, to achieve greater reproductive outcomes, cryopreservation protocols have been continuously refined over the years. In the given section, few of the important animal models has been discussed.

2.7.1 Mouse

Mouse is one of the most commonly used animal model for research. In early 1990s, the first attempt in freezing mouse was a grand success. Later, cryopreservation of mouse spermatozoa resulted in production of a large number of mouse inbred and hybrid strains [75, 76]. There is a growing demand for the production of genetically modified mouse strains since mouse has become the most profound model system to investigate the genetics and pathogenetics of human diseases. Moreover, knock-out projects started in Europe and USA over a decade. As life maintenance of the growing number of mice is difficult and uneconomical in animal laboratories, germplasm cryopreservation provides a valuable means of maintaining transgenic mouse strains used in biomedical research [77]. Moreover, animal gametes- sperm,

eggs, and embryos preservation are now successfully preserved and maintained in cryobanks owing to the cryopreservation technique. Historically, embryo cryopreservation served as the gold standard for maintaining transgenic mice strains with single, multiple mutations, or complex genetic background [78, 79]. However, it is often more expensive due to costly and time-consuming superovulation procedures and subsequent cryopreservation. However, mouse sperm cryopreservation for long-term storage is simple and inexpensive, and it only requires few donor animals for protecting those commonly used inbred strains (e.g., C57BL/6, FVB, and 129/Sv) with single mutations [80]. Thus, sperm cryopreservation provides an efficient management of these genetic resources by reducing maintenance space and cost and by safeguarding them against, for example, disease, breeding failure, and genetic drift.

Although current sperm cryopreservation protocols showed relatively high success, there has been variation in the sperm motility and IVF outcomes post thawing. Many researchers investigated and worked to develop the gold standard protocol for mouse sperm freezing. Nakagata's protocol became the most widely used by many research laboratories and clinical and scientific facilities around the world [80]. The initial freezing solution simply contains 18% dehydrated skim milk and 3% raffinose in water, and cooling is achieved in LN_2 vapor phase for 5 min followed by plunging the samples into LN_2 at −196°C. Since the introduction of this initial protocol, there have been few changes in an effort to improve post-thaw fertilization potential of mouse sperm.

For sperm cryopreservation in mouse, epididymal sperm collected from the cauda epididymis of matured male mice are suspended in sugar-based cryoprotectant, which is loaded in freezing straws and preserved at −196°C [81]. The cryopreserved sperm can be used for efficient fertilization using improved IVF systems featuring methyl-β-cyclodextrin (MBCD) and reduced glutathione (GSH) [82, 83].

Several scientist across the globe had investigated the role of varied antioxidants like monothioglycerol [84], methyl-β-cyclodextrin (MBCD) [82] and the latest refinement was the introduction of reduced glutathione (GSH) to protect spermatozoa against oxidative stress during IVF treatment [83] and increase the fertilization rates. C57BL/6 is a major inbred strain used for the production of transgenic mice, and also as a back-cross for targeted mutant mice [85]. Therefore, it is necessary to establish cryopreservation method for C57BL/6 mouse spermatozoa that could maintain a high fertilizing ability after thawing. Recently, a preincubation medium containing methyl-beta-cyclodextrin used demonstrated increased fertility [82]. Further, it is augmented that sperm freezed in a cryomedia containing 18% raffinose and 3% skim milk, increased fertilization rate [86]. Sperm banking can be used for mice, but in some instances it is also important to bank the female genome [87].

Further, ovarian gamete cryopreservation, as harvested oocytes or contained within the primordial follicles in the cortical patch tissue, can be used for long-term storage of the female germline [88]. Cryopreservation protocol for ovarian tissue based on the slow-cooling procedures was initially developed and used for mouse eggs and embryos in 1970s [10]. This process requires a biological controlled rate freezer or equivalent equipment. It is now documented that in the mouse both fresh and frozen thawed grafts have the potential to restore long-term fertility (i.e., for 1 year) to the graft recipient [89]. A more recent advancement in duration of graft function is in case of ovarian tissue transplanted with transplanted graft functional for 5 years, or more persisting for more than 9 years [90]. Slow freezing is overshadowed with the development of more advanced closed vitrification system, proven more beneficial in ovarian cryopreservation.

2.7.2 Rat

Animal model that is also commonly involved in the scientific work is rat, mainly in basic biology, physiology, brain science, and medicine. Over 40 year ago, the success of IVF was reported in rats. Characteristics, such as a short gestation and a relatively short life span, docile behavior, and ready availability of animals with well-defined health and genetic backgrounds make rat an ideal experimental model. The rat is a standard species for toxicological, teratological, and carcinogenesis testing by the pharmaceutical industry and governmental regulatory agencies [91, 92]. Rats are still continued to be used for nutritional, neurological and endocrinology studies. Cryopreservation has not been performed in rats as often as it has in mice, but the technique is becoming more widespread, for the same reasons that it is used widely in mice. Cryopreservation can be an efficient method of maintaining the potential of raising live mice of the thousands of genetically modified genotypes currently available [93, 94]. Sperm cryopreservation has been similarly successful in rats. Offspring are obtained from thawed sperm using intrauterine insemination or via IVF. Seita *et al.,* [95] established for the first time a successful IVF system using cryopreserved rat sperm [95]. A generalized protocol for IVF in rats (and similar breeds) from cryopreserved sperm and oocytes is illustrated in **Figure 3**.

2.7.3 Rabbit

AI in rabbit using cooled semen stored for short time is a commonest ART practice in country like Europe, where rabbit rearing is common [96, 97]. High fertility rates and proliferation are obtained in this process. However, with limiting factors like low productivity and issues with cryopreserved rabbit sperm, AI is used for experimental or genetic resource bank purposes. Although cryopreserved sperm is not used for commercial purposes at the present, there is a need for reliable methods of rabbit sperm resource banking, especially as this species is a valuable animal

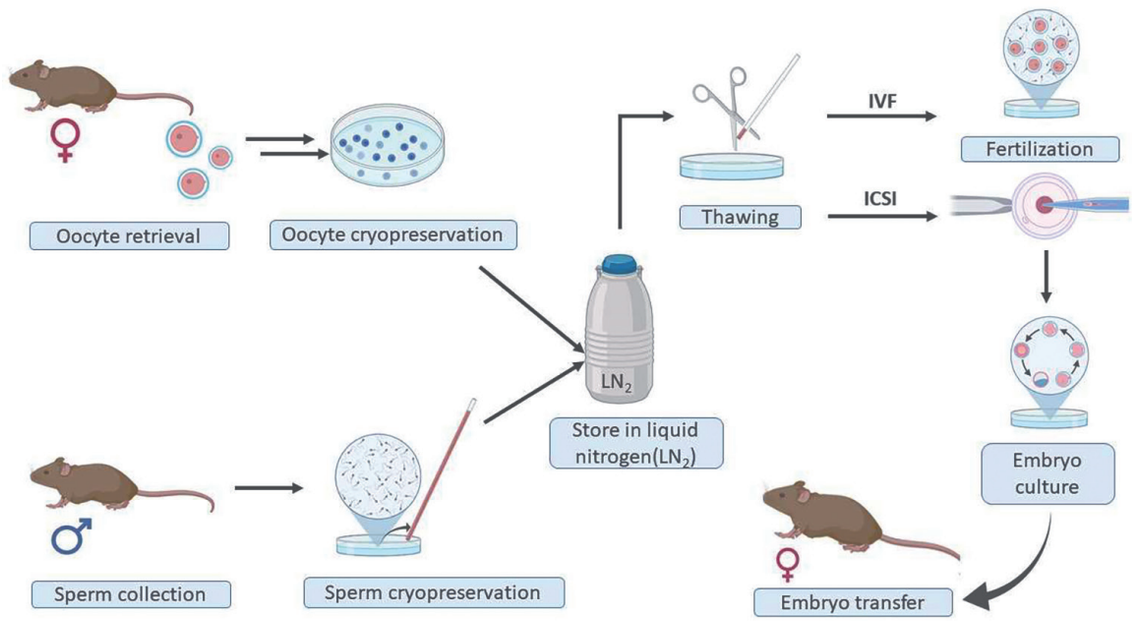

Figure 3.
A schematic representation of Assisted Reproduction techniques and In vitro fertilization; IVF in rats from cryopreserved sperm and oocytes.

in therapeutics (for production of vaccines, antibodies, hormones) [98, 99]. The extent of cryoinjury varies with the species due to differences in the gamete plasma membrane composition among them and cell size as well [100]. Hence, there is no universal protocol followed for all the animal species. It is obligatory to standardize cryopreservation protocol, extender and CPAs levels and composition, for each single species or even breeds.

Researchers observed significantly varying outcomes with the type of CPAs used in cryopreserving rabbit sperm. The use of CPAs like DMSO or ethylene glycol is recommended to enhance the storage of rabbit sperm [101]. Few other studies observed ethylene glycol to be less toxic than glycerol in cryofreezing rabbit sperm. Despite lower toxicity, ethylene glycol failed to provide protection to sperm cells when compared to other DMSO. Importantly, CPAs concentration need to be optimized for sufficient protection. To note, addition of CPAs at 5°C instead of 37°C was reported effective in cryopreserving rabbit sperm as permeability increases with increased temperatures [102]. Data on the CPA type and concentration for optimal cryopreservation is contradictory. However, it is well documented that a balance between all the involved parameters is a key for successful freezing. Sperm quality varies with the type of extenders used. The extenders used for rabbit sperm cryopreservation include a mixture of permeable and non-permeable CPAs. The use of two permeable CPAs is a common practice [103]. Sperm frozen in acetamide extender was found to be of superior quality than sperm DMSO and glycerol or other mixtured extenders [104]. The mixture of CPAs with egg yolk has differential effect on the sperm quality. Sperm frozen in extender containing egg yolk and DMSO demonstrated better sperm quality than for sperm frozen in the extender that contained high DMSO and lacking egg yolk [102]. Hence, it is obvious that a balance of all the essential factors is a key for successful freezing.

Recently, Domingo *et al*., [105] studied comparison of different semen extenders and cryoprotectant agents to enhance cryopreservation of rabbit spermatozoa and found that the addition of dimethylformamide (DMF) to INRA 96® exerts a protective effect on the membrane of spermatozoa improving seminal quality [105]. Although many efforts have been made to optimize cryopreservation extenders and protocols for rabbit sperm, many questions remain unanswered. In addition to cryopreserving sperm, rodents serve as a model for ovarian tissues cryopreservation and transplantation procedures as for current and future application and clinical use in the human. The production of GE rodent models for disease research increased over a decade using gene editing technologies (like CRISPR/Cas9).

2.7.4 Cryopreservation from rodents to larger animals

From rodents to larger animals, cryopreservation is proved to be beneficial in fertility preservation, transplantation and breeding livestock. Advances in cryopreservation pioneered with transplantation of cryopreserved mouse primordial follicles in 1993 by J. Carroll and R. Gosden [106] followed by recognition of sheep as a larger model to study ovarian function. Gosden, in collaboration with D. Baird [107], developed techniques with vascular anastomosis that formed the basis of transplantation of larger organs such as kidney in human. The development of ART techniques has gained significance in the production of commercially important farm animal breeds and a few exotic or endangered species. Livestock industry especially cattle had benefitted to a major extent from the application of cryopreserved semen

or embryos over the past decades. This was also the case in experimenting gamete cryopreservation. Larger animal like sheep is greatly emphasized to study human diseases particularly respiratory diseases and lung cancer, since the anatomy and physiology of the sheep respiratory system is more similar to that of humans than rodents. Sheep has been proposed as a good model for vaccines, asthma pathogenesis and inhalation treatments [108]. The gradual decline of genetic diversity in domestic breeds imposes a major threat to livestock, hence, international community strives harder to conserve the livestock genetics. Semen from most of the mammalian species has been successfully frozen [109].

With regards to temperature tolerance, sperm from different species exhibits varied responses. Bovine sperm shows higher tolerance to low temperatures, while porcine and ovine sperm are more sensitive and at risk of cold shock when exposed to temperature between 5° and 22°C leading to rapid loss of vitality. Animal sperm is highly vulnerable to oxidative damage owing to the loss of antioxidase enzyme and increased fatty acid oxidation on freezing [110]. The stability and viability of sperm is enhanced by adding semen extenders during freezing. The first semen extender for bovine sperm preservation used was egg yolk-sodium citrate diluent (EYC) and was gradually replaced with tris-buffered egg yolk (TRISEY)or tris-fructose yolk-glycerol [111]. Most of the industries use tris and citrate as active components in bovine sperm extenders. Addition of compounds like vitamin E to semen extenders is found to increase the structural integrity of acrosome, and thereby preventing sperm from oxidative damage via its antioxidant properties [24].

2.7.5 Vitrification in larger animals

Vitrification of embryos was invented in 1985 [112]. Later vitrification emerged as one of the powerful methods for cryopreserving embryo from farm animals including cattle, goat, sheep and pig [28, 29, 113, 114]. The birth of the first calf was achieved from frozen/thawed oocyte was reported in 1992 [115]. Vitrification showed high success in bovine oocyte cryopreservation in 1998 [116]. Applicability of macromolecules with lesser toxicity as CPAs, the use of cryotop and solid surface virtification emerged gradually over time to overcome cryoinjury. Treatment with docetaxel improved cryopreservation of bovine oocyte as its protective against cytoskeleton injury thus can potentially enhance survival rate of post thawed oocytes [117]. High rates of survival and development after solid-surface vitrification have been reported for *in vitro* matured oocytes from cows [66] and goats [67]. SSV uses a metal surface, precooled to −180°C by partial immersion into liquid nitrogen, to cool microdrops of vitrification solution containing the embryos or oocytes.

OTC has been reported successful in restoration of ovarian function in sheep model. Complete restoration of acute ovarian function and high rates of natural fertility with multiple live births, were obtained following whole ovary cryopreservation and autotransplantation of adult sheep ovaries [29]. Although ovarian tissue cryopreservation has developed from experiments in sheep in early 1990s, it is now becoming recognized as relatively successful procedure for OTC in human, particularly to preserve the fertility of cancer patients to avoid gonadotoxic damage resulting from the therapy [118]. While there has been considerable success with cryopreservation of oocytes, embryos and semen in farm animals, this technology still requires refinement and further optimizing studies.

3. Conclusion

Based on the utility and need of the animals for varied purposes, fertility preservation is a prerequisite to be practiced in animal husbandry and animal house unit for production of animal models. Understanding of the fundamentals of cryopreservation allows the development of more efficient procedures for cryopreservation of germ cells and further expand their clinical applications and utility in livestock, which can also be transferred to human application. Although, the field of cryobiology has advanced over the years, further research remains required to optimize cryoprotectant concentration, cooling and thawing rates to aim high success in animal reproduction.

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