Genetics and Reproduction

Review article

Current state of slow freezing and vitrification of embryos. Perspectives

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ABSTRACT

Background: Embryo cryopreservation is a technology that enables their storage at low temperatures for extended periods. This technique facilitates the extension of genetic improvement programs and the conservation of endangered breeds and species. **Aim.** To describe the current state of slow freezing and vitrification of livestock-related embryos and their perspectives. **Development:** Due to the current demands of the livestock industry, it is necessary to apply an efficient cryopreservation technique, especially for embryos produced *in vitro*, as they have lower cryotolerance. The most commonly used processes are vitrification and slow freezing, each with its advantages and limitations. **Conclusions:** Vitrification is a cost-effective and fast technique that prevents physical damage caused by ice crystal formation. However, it requires more operator training and is less standardized than slow freezing. Furthermore, the thawing process is better suited to field conditions than devitrification.

Keywords: freezing, cryopreservation, embryos, livestock, vitrification (Source: AGROVOC)

INTRODUCTION

As a result of biotechnological advancements and the economic importance of livestock farming, approximately 1.6 million embryos were produced globally in 2020 among bovine, ovine, caprine, equine, cervid, and camelid species. Of these, 76.2% of embryos obtained from cattle were produced *in vitro*, while figures from other groups using this technique are on the rise (Viana, 2021). Cryopreservation of these embryos allows for their storage without losing their ability to continue development, which enables large-scale transfer programs and commercial use (Cabodevila and Teruel, 2001).

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Despite the trend towards increased production of *in vitro* embryos, a slight decrease in their cryopreservation was observed in 2020. This is attributed to the relatively low survival rates compared to their *in vivo* counterparts (Viana, 2021). Consequently, several studies have focused on improving existing cryopreservation protocols.

The most widely used technologies are slow freezing and vitrification. Both techniques have their advantages and limitations and differ in aspects such as field application, ice crystal formation, and standardization of the technique (Ferré, 2020). The purpose of this review is to describe the current state of slow freezing and vitrification of livestock-related embryos and their perspectives.

DEVELOPMENT

Cryopresercation

Cryopreservation is a technology that enables the preservation of organs, tissues, and cells, such as stem cells, blood cells, sperm, oocytes, and embryos of various species. Furthermore, it allows the storage of biological material for extended periods at a temperature of -196°C, where nitrogen is in a liquid state (Pegg *et al*., 2015). Numerous studies attempted structural and functional suspension and subsequent restoration of these; however, it was not feasible until Polge *et al.* (1949) proposed the inclusion of glycerol in the cryopreservation medium.

The success of this technique lies in its ability to induce and reverse, in a controlled manner, the changes that occur in cells at low temperatures, minimizing the damage during this process (Bojic *et al*., 2021). These processes pose a challenge, as they cause mechanical, toxic, and osmotic changes that affect the cell membrane, mitochondria, endoplasmic reticulum, and genetic expression, increasing cell death (Vining *et al*., 2021; Valente *et al*., 2022; Kurzella *et al*., 2024). Each cell type has a set of optimal conditions determined by the interaction of the cell's unique properties with cryobiological factors (Pegg, 2015).

For example, in embryos, the occurrence of apoptosis as a response to cellular stress negatively impacts the embryo's subsequent ability to hatch, as the expansion of the blastocoel is related to the total number of cells that make up the embryo (Santana *et al.*, 2020). However, an adequate thawing or devitrification protocol results in the regeneration and cellular reorganization of embryonic structures (Estudillo *et al.*, 2021).

Embryo cryopreservation enables genetic improvement, the control of diseases by replacing animal imports, and efficient use of donors and recipients, granting greater flexibility in the availability of the latter (Cabodevila and Teruel, 2001). Moreover, it allows for the establishment of germplasm banks dedicated to conserving endangered breeds and species (Blackburn *et al.*, 2023).

Among the techniques for embryo cryopreservation are slow freezing, vitrification, and ultrarapid freezing. The latter is similar to vitrification but involves intra- and extracellular crystallization. Another technique used is refrigeration, which consists of maintaining embryos at temperatures between 0 and 4°C for up to 72 hours; therefore, it is not considered cryopreservation but rather an intermediate technique between cryopreservation and fresh transfer. Slow freezing and vitrification are the two most widely used processes (Cabodevila and Teruel, 2001).

Slow freezing

The slow freezing technique, also known as conventional freezing, involves embryos reaching osmotic equilibrium before the temperature begins to decrease and maintaining it during cooling. This equilibrium occurs gradually, allowing embryos to contract and release water in response to the increased concentration of the extracellular solution resulting from the addition of cryoprotectants (Cabodevila and Teruel, 2001).

Cryoprotectants increase the solute concentration in the system and facilitate the extrusion of intracellular water, which reduces ice formation and stabilizes cell membranes. These substances are low molecular weight, water-soluble compounds that are classified as intracellular (penetrating the plasma membrane, e.g., glycerol, dimethyl sulfoxide, ethylene glycol, propanediol) or extracellular (e.g., sugars and other synthetic molecules) (Pegg, 2015).

Whittingham *et al*. (1972) were the first to successfully freeze embryos and achieve pregnancies. They exposed mouse embryos to phosphate-buffered saline (PBS) solutions containing dimethyl sulfoxide or glycerol. The temperature was reduced using ice to induce crystallization, and subsequently lowered gradually over specified time periods before the embryos were introduced into liquid nitrogen.

This protocol, also known as the standard method, differs from current protocols by modifying the use of cryoprotectants, cooling rates, and incorporating the "seeding" step. This step is conducted between -4 and -7°C by touching the embryo container with tweezers pre-cooled to -196°C, inducing extracellular ice formation. Freezing methods were later simplified with programmable equipment (Cabodevila and Teruel, 2001).

Various cryoprotectants are used, differing by species and protocol. Whittingham *et al.* (1972) observed higher survival rates in mouse embryos treated with dimethyl sulfoxide. Meanwhile, Barba (2016) achieved better results using ethylene glycol in ovine, bovine, and caprine embryos.

Protocols utilizing ethylene glycol or glycerol with sucrose in bovine embryos enabled direct transfer—a widely used technique in farm settings. This method yields consistently high pregnancy rates (Dochi, 2019). Monosaccharides and disaccharides are frequently used as extracellular cryoprotectants, with sucrose and trehalose being the most commonly employed (Rajan and Matsumura, 2018). Synthetic macromolecules such as hyaluronic acid and polyvinylpyrrolidone are also used, ensuring chemically defined freezing conditions (Barba, 2016). These media can also be supplemented with ascorbate, which reduces intracellular levels of reactive oxygen species and DNA fragmentation (Carrascal *et al*., 2022).

The application of cryoprotectants is primarily limited by toxicity. Thus, recent trends have focused on researching antifreeze proteins, a family of biomolecules naturally produced by various organisms such as yeasts, insects, and fish to sustain life in extremely cold environments (Ekpo *et al*., 2022). The extraction and synthesis of these molecules, and their subsequent use as cryoprotectants, demonstrated effectiveness in semen (Jang *et al*., 2020) and bovine oocytes (Sun *et al.*, 2020), as well as sheep embryos (Li *et al*., 2020; Correia *et al*., 2024). Therefore, the use of biomolecules in embryo cryopreservation across species is considered a promising perspective.

Slow freezing protocols have high standardization and are the most commonly used industrially and commercially. This method mitigates the effects of factors causing cellular damage, such as toxicity, mechanical damage, osmotic stress, and ice crystal formation. The latter, with advancements in protocols, has been minimized to acceptable levels or completely eliminated (Vajta and Kuwayama, 2006).

Vitrification

Vitrification is a technique in which the fluid transitions to a viscous, unstructured solid state similar to glass, hence its name; this is in contrast to freezing, where ice crystals form. A partial dehydration of embryos is performed without allowing them to reach osmotic equilibrium (Cabodevila and Teruel, 2001).

Rall and Fahy (1985) reported the first successful vitrification of mammalian embryos. They balanced mouse embryos in a vitrification solution and exposed them for short periods to increasing percentages of the solution until a reduction in cell volume due to dehydration was observed under the microscope. The solution was formulated with saline and modified with dimethyl sulfoxide, acetamide, propylene glycol, and polyethylene glycol. This study demonstrated that high concentrations of cryoprotectants were required, as minor concentrations resulted in lower embryo survival rates due to intra- and extracellular ice crystal formation.

A disadvantage of vitrification is the cytotoxic effects exerted by these high concentrations of cryoprotectants, even though vitrification protocols are designed to minimize the blastocyst's exposure time to these compounds (Fryc *et al*., 2022). However, this effect can be reduced by appropriately selecting and combining cryoprotectants, which vary depending on the protocol used (Souza *et al.*, 2018), or by incorporating natural substances such as antifreeze proteins.

The most commonly used intracellular cryoprotectants are dimethyl sulfoxide and ethylene glycol (Souza *et al*., 2018; Gómez *et al*., 2020; Najafzadeh *et al*., 2021). Among extracellular cryoprotectants, sugars such as glucose and sucrose are widely employed. These non-penetrating agents create an osmotic gradient that reduces intracellular water content, thereby minimizing the formation of intracellular ice crystals (Rall, 1987). Higher concentrations of ethylene glycol and dimethyl sulfoxide combined with sucrose protect embryos during vitrification better than lower concentrations of these cryoprotectants without sugar (Souza *et al.*, 2018).

Vitrification modulates genes that enhance cryotolerance but may simultaneously deregulate genes critical for embryonic implantation, placental function, cell differentiation, and pregnancy success. It could also impact epigenetic mechanisms regulating apoptotic and necrotic pathways later in fetal development, affecting the survival of vitrified embryos post-transfer (Arshad *et al*., 2021). Studies on vitrified ovine embryos reported high blastocyst recovery rates; however, they had fewer blastomeres than their non-vitrified counterparts (Fryc *et al*., 2022).

Knetter (2023) suggests that cryotolerance percentages in the same species may vary depending on the developmental stage. Stages with fewer cells require vitrification protocols with higher cooling rates. Additionally, the choice of vitrification device can significantly influence blastocyst survival rates and postnatal phenotypic variations (Kim *et al.*, 2020; Garcia *et al*., 2020).

The devices used are classified as fully open in cases where the sample comes into direct contact with liquid nitrogen throughout the entire processing and storage process. Closed-storage open systems, where these are equipped with an additional container that can be sealed prior to storage, so that the sample comes into direct contact with liquid nitrogen during the procedure but not afterward. They are called semi-closed if the sample comes into contact with nitrogen vapors; closed if the support is open and the container is sealed before contact with liquid nitrogen; and lastly, there are sealed straws (Vajta *et al.*, 2015).

In experiments conducted on rabbits, similar development rates were found between fresh embryos and vitrified embryos using Cryotop devices, an open system that loads less than 1µL of vitrification medium, compared to embryos vitrified in mini straws for vitrification (0.125 mL of vitrification medium) (Garcia *et al.*, 2020). Oliveira *et al*. (2020) suggest that an open vitrification device has the advantage of enabling direct warming in the transfer straw.

Recent research has demonstrated that aspirating the fluid present in the blastocoel and the consequent collapse of the embryo prior to performing the vitrification process significantly increases survival rates (Umair *et al*., 2023; Martínez *et al.*, 2024).

Cryopreserved Embryo Transfer

Thawing and devitrification are the reverse processes of freezing and vitrification, respectively. They restore cryopreserved embryos to physiological temperatures. Improper execution of these processes can lead to ice crystal formation or growth, causing mechanical damage and compromising cell viability (Cabodevila and Teruel, 2001).

The thawing speed is determined by the temperature at which slow freezing was halted before immersion in liquid nitrogen. The next step involves removing the cryoprotectant by passing the embryos through drops of medium as necessary or performing direct transfer, depending on the reagents and freezing protocol used (Cabodevila and Teruel, 2001).

Direct transfer's drawback is the inability to assess morphology, often leading to lower pregnancy rates (Cabodevila and Teruel, 2001). However, its primary advantage is implanting more embryos in less time, reducing interference with milk production. Non-direct methods require equipment, an appropriate lab area, and more time (Dochi, 2019).

In the case of devitrification of embryos stored in straws, it is performed by immersing the straws in water at 37°C and then submerging them in the devitrification medium (Vajta *et al.*, 2015). In other vitrification systems, the tip of the device is placed—after previously removing the container in cases where required—directly onto a drop of the appropriate medium to recover the embryos (Canesin *et al.*, 2020).

Devitrification protocols require that embryos be successively washed in different solutions to allow the embryo to re-expand, remove the high concentrations of cryoprotectants—which could cause osmotic shock and affect their survival—and evaluate them before they are transferred. Additionally, this causes the transfer process to take more time (Loutradi *et al*., 2008).

Oliveira *et al*. (2020) proposed a direct transfer method for vitrification, in which an open device is used. This device is introduced with the help of an adapter into a straw containing columns with different concentrations of the medium for devitrification. Although low pregnancy rates are obtained, this protocol is considered promising and is still being refined.

The addition of solutions containing sugars in the thawing or devitrification medium is beneficial for the subsequent development of the embryo. This is due to its non-penetrating characteristics, which allow its effective use in the extraction of highly concentrated cryoprotective agents from blastomeres, regardless of the intracellular cryoprotectant used (Schiewe *et al.*, 2020).

Schiewe *et al*. (2020) reported that a natural product, such as honey—predominantly composed of fructose and glucose—is an efficient, low-cost, and readily available option for any laboratory worldwide to safely extract potentially toxic cryoprotectants from cells before their transfer.

The use of melatonin, a hormone with antioxidant effects, as a cryoprotectant began to be researched in recent years. Studies have determined its regulatory properties on oxidative stress, lipid peroxidation, and DNA fragmentation during embryo vitrification, as well as its use as an additive in the devitrification medium (Marcantonini *et al*., 2022; Choi and Jang, 2022; Li *et al*., 2023). Additionally, melatonin regulates the expression of genes necessary for the future implantation of the embryo (Ivanov *et al*., 2021).

Recent experiments are steering research toward the use of nanomaterials for the administration of cryoprotectants, increasing their penetration while reducing their toxicity. Additionally, the use of magnetic nanoparticles has demonstrated a positive influence during devitrification, preventing the formation of ice crystals during this process (Jones *et al*., 2021; Choi and Jang, 2022; Wang *et al*., 2024; Doultani *et al.*, 2024).

Slow Freezing or Vitrification?

According to the 2021 report by the International Embryo Technology Society (IETS), while there has been a recent trend towards reducing embryos obtained through collection, a significant portion (58.4% of the total produced using this technique) were transferred after cryopreservation. In contrast, only 39.5% of *in vitro*-produced embryos that were transferred were cryopreserved. This is because embryo collection is used as an alternative in situations requiring cryopreservation to compensate for the low cryotolerance of *in vitro*-produced embryos (Viana, 2021), which highlights the importance of selecting the appropriate cryopreservation method in this field.

Vitrification has the advantage of minimizing low-temperature-related injuries since it rapidly passes through critical temperatures. It also prevents physical damage caused by ice crystal formation, a major cause of physical damage to embryos (Rall and Fahy, 1985). This technique temporarily protects a greater proportion of embryos from stress caused by cryopreservation-related damage, resulting in higher embryonic reexpansion, hatching, and *in vitro* survival rates compared to the slow freezing procedure. However, this increased *in vitro* embryonic development does not always correlate with higher post-transfer survival rates (Arshad *et al.*, 2021).

Vitrification is described as a simple method, with the operator's skill and expertise being the most influential factors for success. Unlike slow freezing, vitrification does not require programmable equipment that aids in standardizing the process (Do and Taylor-Robinson, 2019). However, this technique cannot process high embryo volumes due to the multiple equilibration steps involved. Another disadvantage is that current devitrification methods and existing devices are not compatible with direct transfer, a widely used method with slow freezing (Ferré, 2020).

The wide range of vitrification protocols is another aspect to consider, as they differ in timing, temperatures, and cryoprotectant combinations. Additionally, the operator can choose from various vitrification supports (e.g., straws, microdrops, Cryotop, Cryohook, among others). Drop size also varies, which affects medium cooling and devitrification rates (Vajta *et al*., 2015). Consequently, despite being extensively studied, vitrification procedures have yet to be fully standardized, posing a challenge for its application in in vitro embryo production and commercialization (Do *et al*., 2020).

Recently, an automated device has been developed for vitrifying oocytes and embryos. This equipment can transport biological samples, previously loaded into straws, through different vitrification and equilibration solutions at predetermined time intervals, and ultimately immerse them in liquid nitrogen. It can also perform devitrification (Arav *et al*., 2018).

The quality of embryos is another factor to consider when selecting a cryopreservation method. The high lipid content of *in vitro*-produced embryos during the culture period is one factor affecting cryotolerance (Bradley and Swann, 2019). In such cases, vitrification is considered a more suitable technique than slow freezing, as evidenced by higher embryonic survival rates (Bharti *et al*., 2022). Recent studies have focused on reducing the sensitivity of these embryos by adapting culture conditions to reduce lipid accumulation in the cytoplasm through modifications in *in vitro* culture media (de Camargo *et al.*, 2022) or delipidation (Wu and Cheong, 2023). Najafzadeh *et al.* (2021) and Gonzalez *et al*. (2022) also suggest that vitrification is a better alternative than freezing for biopsied embryos.

CONCLUSION

Slow freezing and vitrification are widely used techniques for embryo cryopreservation. While many authors favor vitrification for being a cost-effective and fast technique that prevents physical damage caused by ice crystal formation, it requires more operator training and lacks the high standardization of slow freezing. Additionally, the thawing process is better suited to field conditions than devitrification. Recent research focuses on addressing the main limitations of these techniques, particularly by replacing cryoprotectants with less cytotoxic substances. The suitability of one method or another varies depending on the species being worked with, the working conditions, and the protocols used for obtaining embryos. It is up to the researcher to determine the optimal technique for their laboratory.

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CONFLICTO DE INTERESES

Los autores declaran que no existen conflictos de intereses.