

Cryopreservation by vitrification of *in vitro* obtained bovine oocytes and embryos

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Abstract

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Cryopreservation refers to the preservation of biological material at low temperatures. It applies to various types of somatic cells, tissues, and organs, but also to germplasm, sperm, ova, ovaries, embryos, etc. There are two methods of cryopreservation – slow freezing and vitrification. After the concept of *minimum volume cooling* (MVC) was developed, vitrification became popular. This approach is based on the small sample volume, which leads to ultra-fast freezing, i. e. extremely high rates of cooling and warming. As determining factors for the achievement of vitrification, the speed of cooling/heating and viscosity of the sample are indicated. Nowadays, there is a tendency to reduce or even stop using cryoprotectants by increasing the cooling/warming rate. In many cases, fetal calf serum, albumin, or other substances containing high molecular weight proteins are added to the cryopreservation media.

Keywords: *in vitro*; vitrification; oocytes; embryos; bovine

Introduction

Cryopreservation refers to the preservation of biological material at low temperatures. Generally, cryopreservation refers to the storage of living cells or tissues at temperatures below 0°C, specifically below –80°C, while maintaining functional integrity. Cryopreservation is applied to various types of somatic cells, tissues and organs, germplasm, sperm, ova, ovaries, embryos, etc. Germplasm can be preserved for gene banking purposes or use in human or animal-assisted reproductive technologies (ART), as it allows the preservation of genetic material for a longer time. In domestic animals, embryo cryopreservation has become an important part of breeding programs. Embryos can be thawed at the desired time to be transferred into recipient animals that are on day 6–8 of the cycle after natural estrus.

Cryopreservation of embryos and oocytes has become an important tool for the conservation of endangered wild

species, providing protection against disease, genetic drift, and unexpected losses. For domestic species as well, this method is becoming increasingly important for the preservation of ancient breeds, since in recent decades the genetic diversity of farm animals has rapidly decreased due to changing market demands and agricultural intensification. Approximately 20% of the world's cattle, goat, pig, horse, and poultry breeds are currently threatened with extinction.

There are two methods of cryopreservation – by *slow freezing* or by *vitrification*. Slow cooling leads to intense dehydration in the cell and a strong concentration of intracellular solutes, which can lead to cell damage. On the other hand, when very rapid cooling rates are applied, the cells are not completely dehydrated and the remaining water forms intracellular ice crystals that disrupt the membranes. The main differences between conventional slow freezing and vitrification are summarized in Table 1 and the presented comparison is adapted from Pereira and Marques (2008).

Table 1. Methods for cryopreservation of oocytes and embryos (Adapted from Pereira and Marques, 2008)

Conventional slow freezing method	Vitrification
1. Standard sequins from 0,25 mL	1. Several devices for loading embryos and oocytes (conventional sequins, open pulled sequins, cryoloop, cryoleaf, cryotop)
2. Low concentration of cryoprotectant	2. High concentration of cryoprotectant / reduced volume and time in vitrification solution
3. Controlled slow cooling (0.1 to 1°C/min)	3. Extremely fast cooling rates (-2500°C/min or 20 000°C/min using open drawn paillette and cryoloop)
4. Immersion at -30 to -70 °C and storage in liquid nitrogen (-196 °C)	4. Immersion in liquid nitrogen (-196 °C)

Source: Adapted from Pereira and Marques (2008)

Vitrification of bovine oocytes and embryos

More than a century has passed since vitrification was first described by the German physicist Tammann (1898). He discovered that when a liquid is cooled very rapidly, crystallization can be suppressed by achieving an amorphous „glassy“ state. In the late 1930s, focusing on organic colloids, Luyet (1937) showed how a liquid can achieve a glassy state at extremely low temperatures by modifying its physical properties to preserve cells and avoid damage caused by crystallization.

In the early 1960s, Mazur emphasized that the formation of intracellular ice crystals during cryopreservation was reduced if slow cooling was applied. This is a key factor determining cell survival (Mazur, 1963). He points out that the temperature zone between -10°C and -60°C is critical for cells and that they usually suffer cryodamage under these conditions. Massip et al. (1987) describe vitrification as the solidification of a liquid achieved by an extreme increase in viscosity during cooling so that the solution becomes „glass“. They report the birth of two female calves following vitrified/thawed embryo transfer. This was the first report of viable offspring obtained after vitrification of bovine embryos followed by embryo transfer.

Although vitrification of bovine oocytes remains a challenge, some of the most encouraging results have been in cattle, where progeny have been obtained from immature and mature vitrified oocytes following *in vitro* fertilization and *in vitro* culture (Pereira and Marques, 2008). Vajta et al. (1998) reported obtaining a 25% blastocyst rate on day 8 after vitrification using OPS (*open-pulled straw*), thawing, *in vitro* fertilization, and *in vitro* culture. Germinal vesicle-stage bovine oocytes have homogeneous lipid droplets that show little change after cooling. However, their large size and low surface/volume ratio make it difficult for water and cryoprotectants to move across the plasma membrane (Seidel, 2006). Currently, one of the main problems associated with the cryopreservation of bovine oocytes is the source. The majority of oocytes are recovered from ovaries collected at slaughterhouses, which is a problem because oocytes are

obtained from follicles of different sizes and thus different stages of development, which differ in their cryotolerance. Another limitation associated with this source is the fact that subsequent maturation occurs *in vitro* as opposed to *in vivo*, which also affects the freezing tolerance of the oocytes.

Only quality oocytes should be used to obtain blastocysts suitable for vitrification. Oocytes matured *in vivo* have been shown to have a significantly higher rate of maturation and blastocyst formation than those matured *in vitro* (Rizos et al., 2002). Furthermore, it has been shown that the partial removal of cumulus cells a few hours after the initiation of *in vitro* maturation can facilitate the penetration of cryoprotectants.

Over the past two decades, several vitrification methods have been described by various authors, but it was only after the development of the concept of *minimum volume cooling* (MVC) that vitrification became popular among research groups. This approach is based on the small volume of the sample, which leads to ultra-fast freezing, i.e., extremely high rates of cooling and warming. The development of various vitrification devices allowed high cooling rates to be achieved. These include the 0.25 mL straw (Rall, 1987), the modified minimum drop size (MDS) method (Arav and Zeron, 1997), Hemi-straw (Vanderzwalm et al., 2000), Cryoloop (Lane et al., 1999; Isachenko et al., 2003), the Cryologic Vitrification Method (Lindemans et al., 2003), the Glass Micropipette (Vieira et al., 2007), Hollow Fiber Vitrification (Matsunari et al., 2012) and Rapid-i® (Hashimoto et al., 2013). Several vitrification devices and their corresponding cooling rates are presented in Table 2. These approaches demonstrated the benefits of vitrification in terms of key aspects of cryopreservation: reduction of chilling injury and reduction of the need for high concentrations of cryoprotectant to minimize its toxicity.

Advantages of vitrification for cryopreservation of in vitro-derived oocytes and embryos

Studies on bovine embryos have confirmed the practical applicability of vitrification as an effective cryopreservation

method. A post-thaw survival rate of 80% can be achieved when working with good-quality embryos. One of the most important features of vitrification is that it is a time-saving method and has proven to be as effective as conventional freezing. However, thawing is a critical aspect, and achieving high rewarming rates is necessary to avoid intracellular ice crystal formation.

According to Arav (1992), reducing the sample volume would increase the probability of achieving ice-free cryopreservation. This was supported a few years later by Vajta and Kuwayama (2006), who pointed to two other issues as determining factors in achieving vitrification: *cooling/heating rate* and *sample viscosity*. Many studies have shown the importance of the physical properties of the solution and the rate of decrease in temperature. To characterize these methods developed in the past two decades, a simple classification can be made to categorize them into two groups differentiated by sample volume: those with a large sample volume ($\geq 20 \mu\text{l}$) and those with a small sample volume (MVC, $\leq 3 \mu\text{l}$).

Some other categorizations have been proposed, such as *open* and *closed systems*, in terms of how the sample is subjected to extremely low temperatures. On a pre-cooled surface, in liquid nitrogen (LN_2) (-196°C), nitrogen vapor LN_2 ($\sim -180^\circ\text{C}$) or LN_2 slurry (-210°C) (Yavin et al., 2009), regarding the use of a *carrier for the sample* – with or without a container. In terms of achieved *cooling rate* – slow cooling, rapid cooling, and ultra-rapid cooling methods. The most commonly described in the literature are several vitrification methods, mainly grouped by the minimum volume cooling concept (MVC) – *Cryotop* (Kuwayama, 2007), *open-pulled straw* (OPS) (Vajta et al., 1998) and *Cryoloop* (Lane et al., 1999; Isachenko et al., 2003).

The solid surface vitrification method has shown promising results, as reflected by the survival rate of *in vitro* produced embryos after warming and has the advantage that the

sample does not come into direct contact with liquid nitrogen, avoiding contamination as well. The hollow fiber vitrification (HFV) method (Matsunari et al., 2012) was developed in Japan and applied to the porcine embryos (Machara et al., 2012), mouse embryos (Uchikura et al., 2014) and bovine embryos (Beck et al., 2013).

Although vitrification is easy to perform, saves time, and does not require a programmable freezer, it cannot be overlooked that there are several factors affecting the success rate and application of this cryopreservation technique in practice. Also, although the vitrification of embryos is a time-saving process, thawing can take longer. One possible explanation for this could be that in the early 21st century, research progressed and focused on differences at the molecular level, specifically gene expression profiles and metabolic activity. Leoni et al. (2003) demonstrated that embryos require up to 18 h to fully recover metabolic activity, namely protein secretion, after thawing compared with conventionally frozen embryos.

The importance of achieving ultra-fast cooling and rewarming rates lies in the possibility of reducing the use of cytotoxic cryoprotectants. Previously, experiments were based on very high concentrations of cryoprotectants and slow cooling/warming. Nowadays, there is a tendency to reduce or even stop the use of cryoprotectants by increasing the cooling/warming rate.

Penetrating and non-penetrating cryoprotectants

Embryos and cells are normally in equilibrium with their environment. Solutes are present in the medium, which are required by cells for their metabolism and are actively exchanged to maintain intracellular functions. As stated above, vitrification is a non-equilibrium method and its fundamentals are: ultra-fast cooling/warming rates, minimal sample volume, and increased solution viscosity (namely high so-

Table 2. Vitrification methods

Methods	Authors	$^\circ\text{C min}^{-1}$ *	
Minimum Drop Size (MDS)	Arav and Zeron (1997)	$\sim 180\,000$	Electron-Microscopic (EM) grids
Open Pulled Straw (OPS)	Vajta et al. (1998)	$\sim 20\,000$	Pulled Plastic-Straw
Hemi-straw system	Vanderzwalmen et al. (2000)		Plastic-straw
Cryoloop	(Lane et al., 1999; Isachenko et al., 2003).	$\sim 700\,000$	Nylon loop
Cryologic Vitrification Method	Lindemans et al. (2003)	$\sim 10\,000$	Fiberplug
Cryotop	Kuwayama (2007)	$\sim 40\,000$	Polypropylene strip
0.25 ml Straw	Rall (1987)	~ 2000	Plastic Straw
GPM	Vieira, Forell et al. (2007)		Glass Pulled Micropipettes
Hollow Fiber Vitrification (HFV)	Matsunari et al. (2012)		Hollow Fiber
Rapid-i® Vitrification	Hashimoto et al. (2013)		Rapid-i® straw

*cooling rate ($^\circ\text{C min}^{-1}$)
Source: Author' own elaboration

lution osmolality) due to the use of high concentrations of *penetrating* and *non-penetrating* cryoprotectants.

The efficiency of vitrification as a cryopreservation method has been well documented, but the most important factor that needs to be improved is related to the use of cryoprotectants. Vitrification involves exposure of the embryo to an equilibration and vitrification solution, usually containing increasing concentrations of penetrating and non-penetrating cryoprotectants. During these processes, the embryos undergo dehydration and osmotic stress.

Furthermore, penetrating cryoprotectants enter the cells, while non-penetrating cryoprotectants facilitate dehydration by drawing water out of the cells. Pure water must be cooled at extremely high cooling rates, therefore another function of cryoprotectants is to reduce the cooling rate at which the sample must be cooled to achieve a glassy state.

Penetrating cryoprotectants have the property of forming stable hydrogen bonds with water molecules and proteins. However, their use may be limited by concentration-dependent cytotoxic effects. Some of the most commonly used penetrating cryoprotectants are:

Ethylene Glycol (EG) – is considered a cryoprotectant with minimal toxicity and proven to be the most easily penetrating. It penetrates the cells and reduces the degree of dehydration. It affects the crystallization process by forming undersized crystals. It is used in the cryopreservation of embryos and spermatozoa.

Glycerol (GLY) is the first known and most widely used cryoprotectant. Its mechanism of action is expressed in the binding of intracellular water, dilution of salt solutions, and lowering of the degree of crystallization. Glycerol forms complexes with metal ions and thus protects the active centers of biomolecules from destruction. When cooled, it shrinks and reduces the pressure from the increasing volume of ice on the membrane apparatus of the cells. Its concentration used is critical, as it directly influences toxicity.

Dimethyl sulfoxide (DMSO) is a widely used cryoprotectant. It has good penetrating properties and protects cells from destruction. It has a strong affinity for water and forms stable hydrogen bonds, preventing hyperconcentration of the intracellular solution and influencing the formation and structure of ice crystals. It is used for cryopreservation of embryos and spermatozoa in a concentration of 5–10%. It is used in vitrification media, most often in combination with ethylene glycol and high molecular weight substances.

Propylene glycol or acetamide affects the crystallization process by reducing the size of the ice crystals. Protects cells from osmotic shock and stabilizes plasma membranes. It is widely used to freeze oocytes and embryos at the pronuclear stage.

Non-penetrating cryoprotectants are high molecular-weight substances that do not cross cell membranes. The addition of non-penetrating cryoprotectants to freezing media improves the properties of the solution without increasing its toxicity. The osmotic pressure increases, which helps to dehydrate the cells. Proteins and sugars help to stabilize the membranes, and polymers increase the viscosity of the solution and facilitate vitrification. In this way, the amount of toxic penetrating cryoprotectant required to protect the cells during the freezing process is greatly reduced.

Sucrose – often used in combination with other cryoprotectants. Its presence in the system has a significant osmotic effect. Its addition to the freezing medium dehydrates the cells, thus promoting the formation of smaller intracellular crystals. It stabilizes cell membranes and is used to freeze sensitive objects due to its low toxicity.

Trehalose is a disaccharide with a low molecular mass, but it does not penetrate into cells. It stabilizes cell membranes and plays a key role in protecting the plasmalemma from damage due to freezing dehydration. The exact mechanism of its action is not yet fully understood.

Early studies by Massip (2001) showed the importance of *sucrose* and *trehalose* as key factors in maintaining the functionality and structure of the cell membrane, in an environment of reduced water content. According to Campos-Chillon et al. (2006), *galactose* acts not only as an osmotic agent, but also protects the cell membrane. Adding sugars to the thawing medium acts as a buffer, avoiding the possibility of osmotic shock to the embryo due to extremely rapid rehydration.

Cryoprotectants are used as single components or combined with others to avoid cytotoxicity. Moreover, macromolecules have shown extracellular cryoprotective ability.

Some *synthetic polymers* are also used in cryopreservation:

Ficoll is a nonionic synthetic polymer of sucrose. It is most often used in vitrification solutions, and its addition leads to an increase in the viscosity of the medium. Ficoll 70 (with a molecular mass of 70 kDa) is widely used among its varieties.

Polyvinylpyrrolidone (PVP) is a man-made polymer that has both hydrophilic and hydrophobic properties. In aqueous solutions, its molecules assume a chaotic helical configuration, which allows it to hydrate a sufficient amount of water molecules. Due to its good hydration properties, PVP can lower the crystallization temperature of the aqueous phase as well as reduce ice crystal size. Since its molecules are good complexing agents, they can also affect the osmotic shock that occurs during cooling. It is often used in vitrification solutions, increasing the viscosity of the medium.

Hydroxyethyl starch /HES/ is a polymeric substance containing different molecular weights. It was initially used as a cryoprotectant for erythrocytes, but experience has shown that it is an effective cryoprotectant for other types of cells as well.

Protein macromolecules have been shown to stabilize cell membranes. In many cases, fetal calf serum, albumin, or other substances containing high molecular weight proteins are added to the cryopreservation media. Commonly used penetrating cryoprotectants, non-penetrating cryoprotectants and macromolecular additives employed in vitrification procedures are summarized in Table 3. The classification of cryoprotectants presented in the table is adapted from Moore and Bonilla (2006).

The most important fact related to vitrification is the physical transformation of the liquid medium („solution“) into an amorphous solid state after the glass transition temperature is reached. However, each liquid or solution has its own glass transition temperature, which is estimated at -120°C for standard vitrification media. Several cryoprotectant combinations reported in the literature are summarized in Table 4. These cryoprotectant combinations have been reported by Vajta et al. (1998), Yu et al. (2010), Asgari et al. (2012), Villamil et al. (2012), Trigal et al. (2012) and Sanches et al. (2013).

Damage caused by vitrification

Although vitrification has become the method of choice for IVP embryos, it is known to cause damage at various levels. Morphological, functional, and metabolic changes in

the cells are described. Regarding structural injuries, morphological changes are present in the first hours after thawing. Some authors found disruption of the cytoskeleton, extensive cytoplasmic rearrangement, and changes in the cell membrane of oocytes after exposure to a 4 M solution of EG for 5 minutes. It has been shown to cause damage at the molecular level. A decrease in mitochondrial activity and damage at the ultrastructural level was reported by Dalcin et al. (2013).

Vajta and Kuwayama (2006) showed that intercellular junctions and the cytoskeleton were damaged and attributed these ultrastructural changes to the vitrification process. Although damage was evident 4 hours after thawing, it was barely noticeable 24 hours after thawing. Moreover, the ZP was also changed after the vitrification procedure. A decrease in pore diameter was noted and ZP solidification was observed. Alteration of ZP structure (Moreira da Silva and Metelo, 2005) and ZP cracking (Dinnyés et al., 2000) have been reported in other studies. The use of high concentrations of the cryoprotectant CPA has been cited as one of the major controversies.

Conclusions

Results obtained with bovine embryos have demonstrated that vitrification is an effective cryopreservation method. A post-thaw survival rate of 80% can be achieved when working with good-quality embryos. One of the most important features of vitrification is that it is a time-saving method and has proven to be as good as conventional freezing. However,

Table 3. Penetrating cryoprotectants, non-penetrating cryoprotectants, and macromolecules used in vitrification

Non-penetrating cryoprotectant	Penetrating cryoprotectant	Macromolecular cryoprotectant
Sucrose	EG	PVP
Trehalose	DMSO	BSA
Galactose	1, 2-Propanediol	PVA
Glucose	Glycerol (GLY)	Ficoll 70
Fructose	Propylene glycol	Ficoll 400
Manitol	Acetamide	Dextran

Adapted from Moore and Bonilla (2006)

Table 4. Some combinations of cryoprotectants described in the literature

Methods	Vitrification solutions	Authors
Cryotop	20% EG + 20% DMSO + 0.5M Sucrose	Sanches et al.(2013)
Cryotop	15% EG + 15% DMSO + 0.5 M Sucrose	Asgari et al. (2012)
CVM(Cryologic Vitrification Method)	30% EG + 1 M Trehalose	Villamil et al. (2012)
CVM	16.5% EG + 16.5% DMSO + 0.5 M Sucrose	Trigal et al. (2012)
OPS	16.5% EG + 16.5% DMSO + 0.5 M Sucrose	Vajta et al. (1998)
Closed Pulled Straw	20% EG + 20% DMSO + 18% Ficoll 70 + 0.5 M Sucrose.	Yu et al. (2010)

Source: Author' own elaboration

thawing is a critical aspect, and achieving high rewarming rates is necessary to avoid intracellular ice crystal formation.

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