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REVIEW ARTICLE

STATE OF THE ART OF SLOW FREEZING AND VITRIFICATION OF IMMATURE, MATURE OOCYTES AND EMBRYONIC STAGES

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ABSTRACT

Currently the success through natural fertilization has decreased in humans and domestic species worldwide. In Mexico, subfertility problems have come to affect 1 of 6 couples. According to the World Health Organization, the main causes are: endometriosis (42%), ovulatory disorders (33%), and the increase in the diagnosis of obesity, diabetes and cancer. On the other hand, domestic species of economic importance such as pigs, sheep and cattle have reported numerous fertility problems whose main causes are: 1) low sperm quality due to excessive ejaculations, 2) nutritional deficit, 3) infections, 4) endometriosis, 5) reduced food sources, and 6) heterozygosity loss. Therefore, oocyte and embryo cryopreservation represents an important tool for fertility preservation in the development of assisted reproductive technologies (ART). ART refers to those treatments that can solve natural fertilization problems improving human reproduction and animal production. The importance of cryopreservation is based on its application in patients with ovarian hyper stimulation syndrome diagnosis, cancer, and maternity at advanced age (>40 yr.), for the creation of gene banks and generation of a genetic reservoir in endangered species, such as the Mexican hairless pig and bighorn sheep and finally for basic and applied research. The aim of this review was to analyze two types of cryopreservation methods, slow freezing and vitrification, in terms of its success and impact on different developmental stages.

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INTRODUCTION

Cryopreservation techniques have been applied at different organization levels from organisms, organs, tissues and cells (Katkov, 2012). However, gamete preservation represents a major challenge (Mazur *et al*., 2008) (Table 1).

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Cryopreservation is divided into: freezing and vitrification. In turn, freezing is subdivided into: slow freezing and ultra-rapid freezing. Freezing and vitrification are highly important procedures. However, their effectiveness and application in different developmental stages and cell types have shown different results, increasing the need for further studies to determine its effects (Casillas *et al*., 2015b).

SLOW FREEZING

The first developed cryopreservation technique was the slow freezing about 45 years ago. A study in mice showed that it is possible to recover oocytes after freezing (Whittingham, 1971).

Table 1. Progress of cryopreservation in different species and organization levels

Specie	Oocyte	Embryos	Sperm	Ovarian Tissue	Testicular Tissue
Mouse	+++	++++	+++	++	+
Rat	+	++++	++	++	++
Pig	+	+	++	+	+
Primate	+	++	++	-	++
Fish	-	-	+++	-	-

++++ Good; +++ Acceptable; ++ Regular; + Poor; - None.
Modified from Mazur *et al*., 2008.

This technique involves gamete exposure to low concentrations of cryoprotectants and a gradual temperature (T) decrease. Also, this procedure consists in a freeze-balance transition, due to the exchange of fluids between the intra and extracellular spaces, which are carried out gradual and balanced (Valojerdi *et al.,* 2009). During slow freezing, cells are exposed to T -5 to 7ºC, subsequently to -30 to -65ºC and finally the biological material is stored into liquid nitrogen (LN_2) at -196ºC (Saragusty and Arav, 2011). The cooling rate during slow freezing is 3ºC/min, which could affect viability while the oocyte remains metabolically active (Jain and Paulson, 2006).

Slow freezing has been applied mainly in mature oocyte cryopreservation, reporting the first human birth in 1986 (Chen, 1986). One of the most important advantages of this technique is the use of low concentrations of cryoprotectants, decreasing osmotic and toxic damage in cells. Nevertheless, this has not been enough to reduce intracellular ice crystal formation. Other disadvantages are the procedure time and highly expensive programmable freezing equipment. This is why most studies have focused on increasing the efficiency of this procedure and creating cryopreservation alternatives, such as vitrification (Al-Hasani *et al.,* 2007; Balaban *et al.,* 2008).

VITRIFICATION

The vitrification process consists of transforming a liquid into a glassy vitreous state, excluding ice crystal formation. This technique requires the use of high concentrations of cryoprotectants and high cooling/warming rates to convert the intracellular water into glassy state. The ways to increase the cooling rates are directly related to the process of cellular dehydration before vitrification by cryoprotectants. This event is known as vitreous transition. To achieve this, substances with antifreeze function are added to the cells decreasing the freezing point and preventing ice crystal formation. Since 1962, all improvements in terms of gamete vitrification were unsuccessful (Katkov, 2012). It was not until 1985 that successful vitrification was achieved in 8-cell mouse embryos (Rall and Fahy, 1985). Subsequently, the first human birth from vitrified mature oocytes was achieved in 1999 (Kuleshova *et al.,* 1999). Vitrification involves cell exposure to high concentrations of cryoprotectants and immersion in LN_2. In this way, procedure time during vitrification is highly reduced compared to slow freezing (< 5 sec to 2 h). Also, high cooling rates are achieved by this procedure (20,000ºC/min), preventing ice crystal formation and morphological cell damage (Rojas *et al*., 2004). Other advantages compared to slow freezing are less osmotic damage and no expensive programmable equipment is required (Liebermann *et al.,* 2002). However, some disadvantages are cryoprotectants toxicity (Somfai *et al.,* 2012) and LN_2 storage, since it has been reported that bacteria and viruses can survive at -196ºC. Therefore, potential sterilization methods such as ceramic filters (Cobo *et al.,* 2011) and exposure to ultraviolet light (Parmegiani *et al.,* 2010) are used to eliminate contamination. Despite these disadvantages, several attempts have increased cell recovery after vitrification (Al-Azawi *et al.,* 2013; Casillas *et al*., 2014).

FACTORS AFFECTING OOCYTE AND EMBRYO CRYOPRESERVATION

The main factors are: 1) the permeability coefficient, 2) cryoprotectant concentration, 3) cell carriers, 4) the nuclear stage of the cell, 5) the volume and surface area of the oocytes and embryos, and 6) cooling/warming rates (Saragusty and Arav 2011; Casillas *et al*., 2015a).

CRYOPROTECTANTS

One of the main factors for cryopreservation success is the appropriate selection of cryoprotective agents, whose main function is to protect cells from damage that may be generated during cryopreservation either by slow freezing or vitrification. During cryopreservation, cryoprotectants are in contact with the cells for the intra and extracellular water exchange, causing its dehydration. Cryoprotectants are classified according to their cell permeability. Intracellular cryoprotectants have a low molecular weight and high water solubility, allowing an increased dissemination capacity through the plasma membrane. The main substances with this effect are dimethylsulfoxide (DMSO), ethylene glycol (EG), propanediol and glycerol. Its mechanism of action is the ability to reduce the freezing point and to form hydrogen bonds with water preventing ice crystal formation. Extracellular cryoprotectants are high molecular weight commonly used in mixture with intracellular cryoprotectants to increase dehydration and re-hydration during warming. Other function is to increase the osmolarity in the extracellular space. The main substances with this effect are sucrose, trehalose and glucose. However, other substances such as polyvinylpyrrolidone have been used in vitrification protocols (Mazur, 2004). In comparison with slow freezing, high concentrations of cryoprotectants are required during vitrification (Fahy *et al.,* 1984). Some studies have reported different ways to reduce the cryoprotectant concentration and toxicity. One of the main strategies is the use of a cryoprotectant mixture with EG and DMSO (Fahy *et al.,* 2004; Casillas *et al*., 2014). However, it has also been reported that in bovines the use of this mixture allows to obtain maturation rates up to 41% (Mahmoud *et al*., 2010).

CRYOPROTECTANTS PERMEABILITY

Water is one of the main cellular components and this molecule is capable of enter the cell by simple diffusion. Other type of diffusion was described in 1990, suggesting that small membrane proteins so called as aquaporins can act as channels of water transport (Sales *et al*., 2013). In terms of cryoprotectants, it has also been identified that its dissemination may be through aquaporins, specifically by aquaporin 3 (identified in mouse and bovine morulae and blastocyst) and by the aquaporin 5 in pigs (Sales *et al*., 2013).

Other key factors for cryopreservation success are the developmental stages such as immature, mature oocytes or embryos. This has been explained due to the low cell permeability in early stages. This fact has also been associated with the cell volume and lipid content. Immature oocytes have less intracellular space for cryoprotectant diffusion due to its high lipid content, especially in pigs (McEvoy *et al.,* 2000). In turn, intracellular space is greater in embryos allowing higher rates of cell recovery in comparison with early stages. In addition to the differences between developmental stages, there are also differences between species that determine cryopreservation resistance (Table 2).

Table 2. Cryopreservation resistance among meiotic stages and species

Specie	High resistance	Low resistance
Human	Blastocyst	Immature oocytes
Bovine	Morulae and blastocyst	Immature oocytes
Ovine	Morulae and blastocyst	Immature oocytes
Porcine	Blastocyst	Immature and mature oocytes and morulae.
Equine	Blastocyst	Immature oocytes

According to the previous table description, viability rates after cryopreservation are different among developmental stages and species. Immature and mature oocytes and probably in embryos with 2 to 4 cells, water and cryoprotectants enter to the cell by simple diffusion decreasing viability. For this reason, the way of diffusion plays an important role in cryopreservation success by aquaporins in late developmental stages.

CRYOPRESERVATION CARRIERS

Cryopreservation cell carriers allow the oocytes or embryos to reach a greater cooling rate followed by its rapid immersion into LN_2. Some of the main slow freezing carriers are: Cryostraws (0.5-1 mL) (Lassalle *et al*., 1985), Super Open Pulled Straws (SOPS) (0.25 mL), and Open Pulled Straws (OPS) (0.5 mL) (Vajta, 2000) and for vitrification: Cryoloop (<1 µL) (Lane *et al*., 1999), SOPS, Cryotip (25 µL) (Kuwayama *et al.,* 2005), Cryotop (<0.1 µL) (Kuwayama, 2007), Belved Edge Open Straw (0.25 mL) (Fernandez-Reyes *et al*., 2012), Solid Surface Vitrification (<1 µL) (Somfai *et al.,* 2014) and Cryolock (<0.1 µL) (Casillas *et al*., 2014). Besides an adequate cryoprotectants selection and developmental stage, carriers are essential components to perform cryopreservation. Open and closed carriers have enabled important controversies. Human embryo cryopreservation can only be performed using closed systems to avoid LN_2 contact and contamination. However, closed systems are less effective than open carries because of their low cooling rates and higher volumes.

NUCLEAR STAGE OF THE CELL

Another key cryopreservation factor consists in the nuclear stage of the cell prior to vitrification, where some cell characteristics should be considered:

Immature oocytes at the germinal vesicle (GV) stage

It is well known that oocytes are large cells, in human; its diameter can reach approximately 130 µm. In terms of cryopreservation, the size of the cell is an important factor. Immature oocytes are highly sensitive to low temperatures, which increases ice crystal formation risk (Saragusty and Arav, 2011; Brambillasca *et al.,* 2013). As described previously, oocytes at the GV stage have low permeability to cryoprotectants. In addition, in terms of cryopreservation, they are surrounded by two important barriers: granulosa cells or *corona radiata* (CC) and the zona pellucida (ZP) (Hovarth and Seidel, 2006). Most of the cryopreservation protocols by slow freezing or vitrification of GV oocytes are performed preserving intact oocyte-granulosa cells complex (COCs). This fact has been recently studied because the viability of these somatic cells decreases after vitrification (Casillas *et al*., 2014). These results demonstrate that CC are directly involved in meiotic arrest and resumption. The CC establish communication with the oocyte through gap junctions, where several ions and metabolic precursors are transported for oocyte growth and maturation. Due to the foregoing, it is now suggested that the vitrification of GV oocytes needs to be carried out without the CC, they should be added as a co-culture system to increase maturation (Casillas *et al*., 2014).

In regard to the ZP, it has been described that as a result of slow freezing or vitrification, it can be generated a premature exocytosis of the cortical granules. The permeable cryoprotectants, especially DMSO is able to reach the endoplasmic reticulum and release calcium; as a consequence of this event appears the ZP hardening (Mavrides and Morroll, 2005). Another factor to be considered is that GV oocytes are the stage with the highest lipid content, decreasing cryoprotectants diffusion. It was reported that the main role of the intracellular lipids might be in function of being a metabolic source of energy for the growing cell (Sturmey *et al.,* 2009). However, some studies have focused in diminishing the lipid content through delipidation, centrifugation (Nagashima *et al.,* 1994; Cuello *et al.,* 2010) and assisted laser hatching (Pyror *et al.,* 2011).

Finally, in addition to the size of the oocytes, other factors such as its spherical shape and cell number can affect its viability. In a spherical shape, the dissemination of any substance is difficult to achieve, causing cytoplasm damage. On the other hand, GV oocytes are a unicellular identity compared to the embryos, which have a greater number of cells. In turn, it is reported that embryos can survive and continue their development if at least 50% of the blastomeres are viable (Katkov, 2012). In spite of the difficulties mentioned, a positive aspect compared to those matured or in metaphase II (MII) oocytes is that their chromatin is protected by a nuclear envelop, decreasing the risk of chromosomal alterations caused by cryopreservation (Cooper *et al*., 1998; Isachenko and Nayudu, 1999). For this reason, it was reported the first pig birth derived from vitrified GV oocytes (Somfai *et al.,* 2014). In addition, the vitrification efficiency has also resulted in good quality blastocyst production (Casillas *et al*., 2015a).

Mature oocytes at the MII stage

Although there is a minimal difference between the shape and size of the MII oocytes with those at the GV stage or metaphase I (MI), studies demonstrate that they have a lower

sensitivity to cryopreservation (Somfai *et al.,* 2012). However, the results obtained in MII oocyte vitrification have been controversial because in a greater number of studies meiotic spindle damage is reported, ZP fracture, cortical granules exocytosis and mitochondrial damage (Men *et al*., 2002; Rojas *et al*., 2004; Ghelter *et al.,* 2005). On the other hand, it has been reported that water and cryoprotectant permeability is higher in MII oocytes than GV oocytes, increasing MII cell recovery (Agca *et al.,* 1998). This fact can be explained because MII oocytes no longer require the CC barrier, increasing cryoprotectants permeability. Interestingly, MII oocyte cryopreservation has been applied globally in human assisted reproduction clinics resulting in live births (Porcu *et al.,* 1997). However, despite the high viability rates obtained after cryopreservation, MII oocytes display low blastocyst or embryo development. The only way to produce embryos is suggested by parthenogenetic activation (Ogawa *et al*., 2010). This is mainly due to the fact that during vitrification, ZP hardening occurs decreasing the fertilization potential and as a result, reduced embryo development.

Zygote or two pronuclei stage (2PN)

Slow freezing and vitrification protocols have been carried out in all development stages, including 2PN. Slow freezing is the preferable cryopreservation method in this stage since 1985 with the Cryostraws carrier (Lasalle *et al.,* 1985). In terms of cryopreservation, the 2PN stage is optimal due to the low lipid content. However, in some species, the detection time of the pronuclei formation represents an important limiting factor. Despite this, studies in humans and domestic species have detected PN formation (Payne *et al*., 1997). It is reported that 8 h after fertilization, the 80% of the oocytes display both PN. However, the peak of PN formation and migration occurs until 16 h post-fertilization. At the same time it is important to highlight that 2PN or embryo cryopreservation has been subjected to several ethical, social, moral and religious aspects. So that government and patients in some countries are against cryopreservation procedures, including Mexico. However, in European countries such as Germany, Switzerland and Italy, the embryo protection legislation has established that embryo cryopreservation is possible to achieve only at the 2PN stage because syngamy has not yet been reached (ESHRE, 2001). Despite the high slow freezing efficiency, currently, vitrification has proved to be more efficient. Pregnancies and births have been reported (Kuwayama *et al.,* 2005), but also this success has been achieved in domestic species as the pig (Somfai *et al.,* 2009).

8 cell embryo and morulae stages

Embryo cryopreservation represents an important advance for ART. Currently its application in IVF cycles and ICSI allows obtaining a greater number of embryos, decreasing the risk of ovarian hyperstimulation syndrome in patients subjected to several hormonal stimulation cycles. In turn, a greater number of embryos can be transferred in time, ensuring implantation rates. Births by slow freezing (Zeilmaker *et al.,* 1984) and vitrification (Mukaida *et al.,* 1998) can be obtained after8 cell embryo cryopreservation.

Table 3. Percentages of viability anddevelopment post-vitrification in different meiotic stages and species

Specie	Nuclear stage	Viability (V) or development (D) rate after vitrification (%)	Reference
Human	MII	56% V	(Criado *et al*., 2010)
	Blastocyst	96% V	(Hiraoka *et al*., 2004)
Mouse	MII	45% V	(Lee *et al*., 2010)
	4 Cell	50% V	(Lee *et al*., 2007)
	Blastocyst	10% V	(Yavin *et al.,* 2009)
Porcine	Blastocyst GV	62-77% V	(Cuello *et al*., 2004)
		96% V and 62% D	(Casillas *et al*., 2014; Casillas *et al*., 2015a)
Ovine	GV	25% V	(Isachenko *et al.,* 2001)
Bovine	MII	40% D	(Martino *et al*., 1996)
		28% D	(Arav y Zeron, 1997)
		39% V	(Santos *et al*., 2006)

Table 4. Live births by different cryopreservation methods

Specie	Cryopreservation method	Nuclear stage	Reference
Human	Vitrification	MII	(Smith *et al*., 2010)
		Blastocyst	(Aflatoonian *et al.,* 2010)
Bovine	Slow Freezing	MII	(Fuku *et al*., 1992)
		GV	(Vieira *et al*., 2002)
Porcine	Vitrification	MII	(Li *et al*., 2006)
	Vitrification	GV	(Somfai *et al*., 2014)
Equine	Vitrification	MII	(Maclellan *et al.,* 2002)

Table 5. Comparison of the slow freezing versus vitrification efficiency in the viability and fertilization of MII oocytes in humans

	Slow freezing (%)	Vitrification (%)
Number of assisted reproduction cycles	30	48
Cryopreserved MII oocytes	238	349
Viability post cryopreservation	159/238 (67)	281/349 (81)
Viability 4 h post cryopreservation	155/238 (65)	260/349 (75)
Fertilization	104/155 (67)	200/260 (77)
Pregnancies per cycle	4/30 (13)	18/48 (38)

Other embryo cryopreservation benefits are: 1) the generation of cell banking for other couples, 2) the possibility to detect genetic abnormalities and diseases, and 3) embryos that are not used in the first ART cycle, provide the couple more opportunities of pregnancy in further cycles.In terms of cryoprotectants permeability, 8 cell embryos have less intracellular lipids increasing viability rates. However, it has been established that this stage of development is critical since the segmentation of each blastomere is able to give rise to another intact embryo; this means that blastomeres are totipotential. This is why these stage embryos are mainly used for cloning technologies. Also, before morulae formation, embryo undergoes a compaction process, forming excessive permeable sites, which could damage the embryo. In morulae stage, high viability percentages are obtained after cryopreservation (74%) (Cremades *et al*., 2004). In this stage, one of the most important benefits consists in the greater cell number (16-32 cells), allowing cryopreservation resistance. As it has been indicated in this review, the lipid content decreases in the embryo stages compared to GV, MI or MII oocytes so that, in morulae, cryoprotectant permeability is a minimum problem (Pyror *et al.,* 2011).

Blastocyst stage

When the embryos at the morulae stage fall into the uterine cavity after approximately 4 days post-fertilization, they form a cavity in the embryonic mass that is known as blastocele. Fluids from the uterine cavity go through the ZP to inside the cell, allowing the separation of the inner cell mass (originating the embryo) and trophectoderm (originating the placenta). When this separation occurs is known as blastocyst. In terms of cryopreservation, the blastocyst stage represents high advantages; they have a larger cell number up to 120 blastomeres so that its loss does not represent a limiting factor. In addition, the ZP becomes thin allowing a greater permeability. However, its size and blastocele makes them highly sensitive to any type of manipulation. One of the main cryopreservation problems is an inadequate dehydration allowing ice crystal formation because of the large amount of water in the blastocele. However, to minimize this damage, blastocele punction before cryopreservation is carried out. This punction consists in the preparation of a glass pipette followed by the blastocyst puncture by the ICSI microscope (Hiraoka *et al.,* 2004). Currently the best technique reported to cryopreserve this stage is vitrification with high viability rates in species such as humans and pigs (Table 3).

As important to be mentioned, the first human blastocyst vitrification was reported by Cohen *et al*. (1985). However, to date, several factors are involved in blastocyst cryopreservation success; mainly the use of high concentrations of cryoprotectant has allowed dehydration with less morphological damage. Also, the metabolic requirements of the blastocyst are greater; they require a high glucose consumption, which is added as a cryoprotectant, so that glucose does not represent toxicity in this stage. In addition, carriers such as Cryotop and Cryolock are designed mainly for embryo cryopreservation and storage. The effectiveness of the vitrification versus slow freezing is well documented. Slow freezing attempts are registered 13 years before vitrification creation. Both procedures have demonstrated similar pregnancy and birth rates in humans (Aflatoonian *et al.,* 2010; Smith *et al*., 2010). However, the number of births by vitrification has increased significantly compared to slow freezing in humans and domestic species (Table 4). Also in terms of vitrification, clear evidence is shownthat vitrification is superior to slow freezing (Table 5). Meta-analyzes studies data support that vitrification is now the preferable cryopreservation procedure for ART (AbdelHafez *et al.,* 2010; Edgar and Gook, 2012).

Conclusion

The use of slow freezing for oocyte and embryo cryopreservation has decreased significantly and in most fertilization centers its use has been completely replaced by vitrification. Vitrification has increased its practice worldwide and is now considered as the present and future of cryopreservation. As mentioned at the beginning of this review, the vitrification procedure is a simple method with low cost and is based on the use of high concentrations of cryoprotectant and high cooling rates that ensures the transition of the cell to a vitreous state, excluding ice crystal formation in comparison with slow freezing. Oocyte cryopreservation has proved less efficiency compared to embryonic stages. However, more studies are still required to increase the effectiveness of the cryopreservation procedures and also to report if there is future damage in the offspring. In spite of the fact that there are only few studies, it has been reported that so far there is no evidence that the use of vitrificationcan cause an adverse effect on the health of children conceived by these procedure (Wennerholm *et al.,* 1998). This allows the application of the vitrification procedure in all the species that need to improve their reproductive capacity.

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