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Review Article

Immature Oocytes Viz-a-Viz Vitrification for Long Term Female Germplasm Preservation: An Overview

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Abstract

Germplasm cryopreservation is an important ex situ conservation programme to preserve the important animal genetic resources for future use. Cryopreservation processes used for mammalian cells include slow freezing and vitrification with recent shift towards vitrification especially in case of oocytes and embryos as it produces less damage to the oocytes and embryos than slow freezing. Vitrification of oocytes has almost become a standard approach for cryopreservation of female gametes due to numerous efforts like development of novel cryodevice such as OPS or Cryotop which resulted in increasing cooling and warming rates thereby cryosurvival and blastocyst yields of vitrified oocytes. Now attempts are focused on the qualitative improvement of oocytes prior to the vitrification in order to increase their revivability to acceptable levels, to overcome the high sensitivity of oocytes to cryopreservation and to improve blastocyst yield and pregnancy rate. This article is an overview of immature oocytes vitrification and its various aspects as a tool for long term preservation of female germplasm.

Keywords: Female Germplasm, Immature Oocytes, Preservation, Vitrification

Introduction

Cryopreservation is the use of very low temperatures to preserve structurally intact living cells and tissues for a long period of time (Pegg, 2007). Depending on the cell types or given cells among different mammalian species, there is great diversity in cryobiological response and cryosurvival during the freezing and thawing cycle (Gao and Critser, 2000). Germplasm cryopreservation is considered an important ex situ conservation programme to preserve the important animal genetic resources for future use. Germplasm cryopreservation has become an indispensable rescue strategy to preserve biodiversity and to facilitate the natural processes of evolution to continue in case of threatened species and natural disasters especially when in vitro embryo production by in vitro maturation (IVM) and in vitro fertilization (IVF) using immature oocytes and frozen-thawed spermatozoa has become a routine practice (Wright *et al*., 1976; Wright. 1977). Cryopreservation processes used for mammalian cells include slow freezing and vitrification with recent shift towards vitrification especially in case of oocytes and embryo cryopreservation as it produces less damage to the oocytes and embryos than slow freezing (Kuleshova and Lopata, 2002). The major steps involved in cryopreservation process of cells include mixing of cryoprotective agents (CPAs) with cells or tissues before cooling, cooling of the cells or tissues to a low temperature and its storage and finally warming of the cells or tissues and removal of CPAs from the cells or tissues (Gao *et al*., 2016). However, the appropriate use of CPAs is important to improve the viability of the sample to be cryopreserved.

Vitrification process which is actually transformation of a substance into a non-crystalline amorphous solid involves rapid cooling of a liquid so that it passes through the glass transition to form a vitrified solid. In vitrification process, there is dehydration of cells like oocytes or embryos by exposing briefly to a concentrated solution of cryoprotectants before the samples are plunged directly into liquid nitrogen. By the end of the 1990s, vitrification was applied to human embryos achieving live births with both blastocyst and cleavage stage embryos (Hsieh *et al*., 1999; Yokota *et al*., 2000) but still the vitrification of oocytes was lagging because of lack of appropriate carriers. However, the introduction of Cryotop in Japan as a suitable carrier for oocytes marked the breakthrough that lead to the adoption of vitrification of oocytes into routine clinical practice. It allowed for an extremely rapid cooling rate that was facilitated by a minimal volume and resulted in a very high survival rate and live births (Katayama *et al*., 2003; Kuwayama *et al*., 2005; Kyono *et al*., 2005). Presently, different cryoprotectants like ethylene glycol (EG), glycerol (GLY), dimethylsulfoxide (DMSO), propylene glycol and 1,2-propanediol (PROH) have been used in different combinations for vitrification of mammalian oocytes. Vitrification focuses on the total elimination of ice crystal formation in both the extra- and intracellular solutions and this goal is usually achieved by transferring the oocytes to a solution with a relatively high concentration of cryoprotectants, as well as by using extremely high cooling and warming rates and by using small (<1 ml) solution volumes that are exposed directly to liquid nitrogen. The small volume also prevents heterogeneous ice crystal formation, and the high cooling and warming rate at relatively high temperatures decreases chilling injuries. To attain high cooling rates, some devices and methods have been designed to reduce the volume of vitrification solutions, such as electron microscope grids (EMG), cryotop, cryoloop, solid surface, nylon mesh, cryoleaf, open pulled straw (OPS), closed pulled straw (CPS) and flexipet-denuding pipette etc.

Immature Oocyte Preservation

Cryopreservation of gametes and embryos has become an integral part of assisted reproduction technologies (ART). Embryo cryopreservation has also become a routine practice in clinical ART and has resulted in delivery of many healthy babies (Loutradi *et al*., 2008). However, embryo cryopreservation has some drawbacks; it requires the availability of male partner to produce those embryos, in addition, embryo cryopreservation is prohibited due to ethical, legal and religious implications in some countries (Chian *et al*., 2009). Cryopreservation of unfertilized oocytes is an alternative option, giving flexibility during in vitro fertilization (IVF) and a potential to establish oocyte banking with oocyte donation (Nagy *et al*., 2009; Noyes *et al*., 2010; Noyes *et al*., 2011; Cobo *et al*., 2017). Oocyte cryopreservation is important for the establishment and maintenance of genetic banks of female animal genetic resources of importance for future use. Cryopreservation of oocytes through vitrification is widely practiced owing to its uncomplicated procedure, higher survivability of oocytes and embryos and yielding higher pregnancy rates (Garcia-Velasco *et al*., 2013). The majority of successful assisted pregnancies to date have involved frozen and subsequently thawed mature oocytes that were collected after ovarian stimulation or in vitro maturation. Recently, numerous studies have been conducted to cryopreserve GV-oocytes in many mammalian species (Vieira *et al*., 2002; Somfai *et al*., 2007; Vieira *et al*., 2008; Moawad *et al*., 2011; Moawad *et al*., 2012; Moawad *et al*., 2013b; Kim *et al*., 2014; Moawad *et al*., 2017) and reportedly produced live births in humans and animals (Tucker

et al., 1998; Aono *et al.*, 2005, Vieira *et al*., 2008), although the blastocyst development rate remains low (Moawad *et al*., 2013b; Moawad *et al*., 2017; Argyle *et al*., 2016). Vitrification of oocytes at metaphase II (MII) stage can disrupt the meiotic spindle (Gomes *et al*., 2008) which could be avoided by freezing of oocytes at the germinal vesicle (GV) stages in which chromosomes are inactive, and the complete barrel-shaped spindle has not formed yet (Cooper *et al*., 1998; Isachenko and Nayudu, 1999). Immature oocytes are arrested in the diplotene stage of prophase I with diffuse chromosomes surrounded by a nuclear membrane and hence, reduces the risk of polyploidy and aneuploidy (Cooper *et al*., 1998; Isachenko *et al*., 1999). Immature oocytes are with homogenous lipid droplets that show little change following cooling. Communication between cumulus cells and the GV-stage oocytes by intercellular coupling via gap junctions may be sensitive to osmotic stress, so it is important to maintain the functional integrity of the cumulus cells after vitrification and warming to obtain cytoplasmically matured oocytes following IVM process. Thus, freezing of oocytes at GV stage has many clinical advantages in human and animal reproduction (Brambillasca *et al*., 2013; Moawad *et al*., 2011; Moawad *et al*., 2013a; Moawad *et al*., 2013b). However, germinal vesicle stage oocyte freezing is reported to impair subsequent development up to the blastocyst stage following maturation and fertilization after thawing (Toth *et al*., 1994; Son *et al*., 1996). Vajta *et al*. (1998) reported that 25% of bovine GV oocytes vitrified-warmed using OPS system could develop into the blastocyst stage on Day 8 but it is highly significant only when the high revivability of post-warm GV oocytes in the OPS system is reproducible, (Vieira *et al*., 2002). Abe *et al*. (2005) reported that 8% of bovine oocytes vitrified-warmed on nylon-mesh holder as a cryodevice developed into blastocysts when they were exposed to EG + Ficoll + sucrose-based solution in a stepwise manner with a live calf after transfer. Difficulties still exist with in vitro maturation (IVM) of GV stage oocytes after freezing and thawing procedures. No standard IVM protocol for GV-oocytes has yet been established in humans and in some animal species (Shahedi *et al*., 2013; El-Shalofy *et al*., 2017). Further, the sensitivity of oocytes membranes to ice-crystal formation, and that of the cytoskeleton fibers to low temperatures and cryoprotectants, is frequently associated with substantially reduced viability of frozen-thawed oocytes (Liu *et al*., 2003) but survival may be increased after vitrification or ultrarapid freezing because high cooling rates circumvent the chilling sensitivity of this cell type (Martino *et al*., 1996) as vitrification can effectively prevent intercellular ice-crystal formation and decrease chilling injury (Kuleshova and Lopata, 2002). Therefore, further study in detail is needed to enhance the developmental potential of vitrified GV oocytes after thawing in order to achieve good clinical results.

High Cryosensitivity of Oocytes

Cryopreservation of immature oocytes at the GV stage is considered a subject for challenging endeavour. The size of oocytes is much larger with a small surface to volume ratio than the sperm and even the blastomeres of an early embryo (Chen *et al*., 2003) which leads to difficulty in achieving dehydration and penetration of CPA during cryopreservation process. Furthermore, movement of water and CPAs is affected partially due to the lack of aquaporin expression and difference in the plasma membranes of oocytes from those of embryos (Jin *et al*., 2011). There is a rise of intracellular free calcium during fertilization, which makes the ionic strength and membrane potential of the plasma membrane (Gook *et al*., 1993). Although cell viability and functional state may be preserved for long terms (Mazur, 1970) at very low sub-zero temperature but some physical stresses can damage cells at the various sub-zero temperatures. Intracellular ice formation is a major factor to cause cell damage and hence various approaches like increasing concentration of cryoprotectants in combination, reducing volume and increasing cooling rate in an attempt to avoid cell damages (Saragusty and Arav, 2011). Increasing the concentration of CPA lead to osmotic injury and toxicity and incidence of cryo-injuries depends on various factors like size and shape of the cell, the permeability of the cell membranes, and the quality of the cells and even species, developmental stage, and origin (Vajta and Kuwayama, 2006). Although, there are reports of offspring born from various species using frozen-thawed oocytes but the embryo development rate is still low following cryopreservation procedures due to susceptibility of oocytes to damage during cooling and/or freezing and subsequent thawing, fracture damage in zona pellucida during cryopreservation procedures and damage to intercellular communication via gap junctions between cumulus cells and the oocytes (Fuku *et al*., 1995; Hochi *et al*., 1996; Lane *et al*., 1999). In addition, there is zona hardening due to premature release of cortical granules, spindle disorganization and loss or clumping of microtubules resulting in low fertilization rates of cryopreserved oocytes (Carrol *et al*., 1990; Aman and Parks, 1994; Fuku *et al*., 1995). Further, there is transient rise of intracellular free calcium following exposure of mature oocytes to CPA and/or chilling procedure preventing the sperm entry into the oocytes (Morato *et al*., 2008; Rojas *et al*., 2004; Succu *et al*., 2007; Pereira and Marques, 2008). Further, damage to the meiotic spindle, actin filaments, chromosomal dispersal and microtubule depolymerization and multiple aster formation in vitrified-warmed oocytes (Massip, 2003; Ogawa *et al*., 2010; Hara *et al*. 2012) may be related to loss of ooplasmic function responsible for

normal microtubule assembly and ultimately decrease in developmental competence of vitrified-warmed oocytes.

Duration of Oocyte Storage

Oocytes stored at the temperature of LN2 (−196ºC) at which the biological activities of cells are stopped completely are supposed to be relatively safe without any obvious undesirable biological and metabolic changes in the ooplasm. So far, most studies regarding quality of embryos cryostored at−196ºC indicated that the quality of embryos is not influenced by the duration during cryostorage (Whittingham *et al*., 1977; Glenister *et al*., 1984; Glenister *et al*., 1986). However, reports on oocyte cryopreservation are of a relatively short duration in LN2 before warming. Results of live birth rates of human oocytes stored in LN2 by a slow freezing method with a plastic straw revealed that oocytes can be safely cryopreserved for several years, (Parmegian *et al*., 2009) with a recent report of a live twin birth after IVF of oocytes cryopreserved for almost 12 years (Quintans *et al*., 2012). Still evidence provided do not lend support for the practice of oocytes cryobanking requiring a long duration of cryostorage (Coticchio *et al*., 2005) as cryosurvival, fertilization rate and embryonic development of the vitrified mouse oocytes has been found affected significantly, in an adverse manner, by the cryo-storage duration in LN2 (Yan *et al*., 2011). Further, many important photo-physical events or chemical synthesis occur to produce some form of hazards in LN2 (McGee and Martin, 1962). Formation of free radicals, production of breaks in macromolecules and oxides of nitrogen in LN2, which enhances the yield of ozone by a catalytic effect (Brereton, 1988; Gregory and Nuttall, 1995) are a source of damage at such low temperatures ozone production may deleterious damage DNA and cause DNA breaks since an enzymatic repair cannot occur in LN2 (Rice, 1960). So, effect of cryo-storage duration on subsequent oocytes survival, fertilization as well as pregnancy rates after warming needs further study in order to draw any conclusion.

Recent Improvement of Oocyte Cryosurvival

Regarding oocytes vitrification, it appears that the recent focus has been shifted from modifying the CPA composition, the cryodevice, or the CPA addition/dilution process in the vitrification to procedures like delipidation and chemical treatments/supplementation of oocytes prior to vitrification, during the recovery culture and during IVM after vitrification. Partial removal of cytoplasmic lipid droplets partially in bovine oocytes (Otoi *et al*., 1997) significantly inhibited polyspermic penetration in vitrified-warmed oocytes (blastocyst yield; 11% versus 7% in non-centrifuged control). L-Carnitine, an active form of carnitine, can enhance lipid metabolism in animal cells and play an important role in the transportation of fatty acids from the cytoplasm to the mitochondria for β-oxidation (Kerner *et al*., 2007). Hence, L-carnitine can enhance ATP production in animal cells (Vanella *et al*., 2000) and stimulate mitochondrial metabolism during maturation as previously reported in mouse oocytes (Dunning *et al*., 2010). Supplementation of L-carnitine into IVM medium for cytoplasmic lipid droplets rich porcine oocytes reduced the amount of the lipid droplets and changed their distribution from the cortex to the medulla of oocyte cytoplasm (Somfai *et al*., 2011) increased cryotolerance and developmental competence of bovine embryos cultured in L-carnitine supplemented medium (Takahashi *et al*., 2013). Researchers (Chankitisakul, *et al*., 2013; Phongnimitr *et al*., 2013) described the effect of L-carnitine supplementation into IVM medium for bovine oocytes on their cryotolerance. Chankitisakul *et al*. (2013) showed that bovine oocytes matured in the presence of L-carnitine (0.6mg/ml) had higher developmental potential to blastocyst stage 8 days after vitrification and IVF when compared to those matured in the absence of the L-carnitine (34% versus 20%, fresh control; 44%). Glutathione plays an important role in protecting cells against the destructive effects of reactive oxygen species (ROS) and GSH levels of IVM oocytes are lower when compared with those of ovulated oocytes, as reported in some species (Brad *et al*., 2003; Rodrıguez-Gonzalez *et al*., 2003; Kim *et al*., 2007; Ge *et al*., 2008). Supplementation of β-mercaptoethanol and cysteamine into IVM medium can increase intracellular GSH level, IVM rate and the developmental potential of the bovine oocytes (de Matos *et al*., 1996; Sofi *et al*., 2011). Oocyte vitrification procedure increase apoptosis of embryonic stem cells results in a decrease of developmental competence (Morato *et al*., 2010; Li *et al*., 2012). Inhibition of the Rho-associated coiled-coil kinase (ROCK), a downstream target of the small GTP-binding protein Rho (Matsui *et al*., 1996) has been found involved in decrease of apoptosis in embryonic stem cell derived neural cells (Koyanagi *et al*., 2009) and the revivability of in vitro produced bovine blastocysts after vitrification and warming (Hochi *et al*., 2010; Hwang *et al*., 2013). Interestingly, the supplementation of α-tocopherol, not ascorbic acid, to the recovery culture medium resulted in a significantly higher blastocyst yield from the post warm oocytes as 37% versus 26% in the post warm control oocytes (fresh control; 53%) (unpublished data of I. Yashiro and S. Hochi). Antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs), collectively referred to as AF(G)Ps, have ice-binding affinities and can kinetically suppress the temperature for ice-crystal formation, thus preventing

mechanical cell damage caused by ice regrowth (Jia and Davies, 2002). Antifreeze protein supplementation of the vitrification medium has a protective effect on immature oocytes, promoting their resistance to chilling injury, may preserve spindle forming ability and membrane integrity at GV stage (Jo *et al*., 2012). Recently, improved survival rates have been reported in animal models using modified vitrification procedures, particularly those involving the use of AFPs-supplemented freezing and thawing solutions (Makarevich *et al*., 2010). Antifreeze glycoprotein-8 (AFGP8) supplementation during vitrification effectively protected bovine MII-stage oocytes against chilling injury, as well as improves their cryosurvival and subsequent embryonic development (Liang *et al*., 2016). Supplementation of AFGP8 during vitrification resulted in significantly higher rates of normal spindle organization and chromosome alignment, actin filament impairment, and mitochondrial distribution (Liang *et al*., 2016).

Natural honey is a mixture of sugars along with traces of other bioactive substances such as organic acids, enzymes, antioxidants and vitamins. Presence of variety of amino acids like glycine and alanine help cell membrane from freezing by stabilizing phospholipids (Anchordoguy *et al.,* 1988). Natural honey has antimicrobial (antiviral, antibacterial and antifungal), antioxidant, antitoxin, anti-inflammatory, antimutagenic, anticancer and immunosuppressive properties (Pulcini *et al.,* 2004). Addition of glutamine, one of the amino acids in honey, to vitrification solution improved the maturation ability of vitrified-warmed immature bovine oocytes (Yamada *et al*., 2011) and natural honey has been recently used as a non-permeating CP to dehydrate bovine oocytes for vitrification (Alfoteisy *et al*., 2020). Addition of 10 mM caffeine during the last 6 h of IVM failed to restore the spindle integrities and subsequent development of vitrified/warmed oocytes at the germinal vesicle stage (Moawad *et al.,* 2018). Cytoplasmic maturation of oocytes manifested by spindle assembly, chromosomal alignment, mitochondrial distribution and blastocyst development following in vitro fertilization was significantly improved on addition of glycine in either vitrification/thawing or maturation medium (Cao *et al.,* 2016). Arcarons *et al.* (2017) observed that CLC (cholesterol-loaded methyl-β-cyclodextrin) pre-treatment before the vitrification of bovine GV or *in vitro* matured oocytes does not appear to affect subsequent cleavage and embryo development rates. Synthetic ice blockers like SupercoolX-1000 have been used in vitrification medium to improve cryosurvival and developmental rates of oocytes in mice (Fahy *et al*., 2004) and horses (de Leon *et al*., 2012). Supplementation of SupercoolX-1000 in vitrification medium for immature porcine oocytes improved the ability of surviving oocytes to cleave but not to develop into blastocysts (Santose *et al*., 2017). Above studies thus demonstrate that high rates of oocyte survival can be achieved after vitrification of oocytes even at the GV stage by careful optimization of a cryoprotectant treatment (CPA) regimen (Somfai *et al*., 2015).

Conclusion

Vitrification of oocytes has almost become a standard approach for cryopreservation of female gametes due to numerous efforts like development of novel cryodevice such as OPS or Cryotop which resulted in increasing cooling and warming rates thereby cryosurvival and blastocyst yields of vitrified oocytes. Now attempts are focused on the qualitative improvement of oocytes prior to the vitrification and the short-term recovery culture of vitrified-warmed oocytes by supplementation like L-carnitine, ROCK inhibitor, cysteamine, caffeine etc. prior to the subsequent IVF. In addition, chemical treatment of oocytes before or after the vitrification protocol can make it possible to increase their revivability to acceptable levels. Further improvements of the vitrification procedure, combined with pre- and post-vitrification treatment/supplementation may help to overcome the high sensitivity of oocytes to cryopreservation and to improve blastocyst yield and pregnancy rate. Such knowledge will be essential for optimization of the current vitrification protocol in order to minimize cryoinjuries during vitrification of immature oocytes and to identify the reasons for reduced developmental competence to the blastocyst stage in surviving and cleaved oocytes.

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Conflict of Interests

There is no conflict of interest.

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