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REVIEW

What does the cryopreserved oocyte look like? A fresh look at the characteristic oocyte features following cryopreservation

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Abstract In October 2012, the American Society for Reproductive Medicine (ASRM) and, in March 2012, the European Society of Human Reproduction and Embryology (ESHRE), lifted the categorization of oocyte cryopreservation as being "experimental" and endorsed its entrance into the mainstream of assisted reproductive techniques. This change in policy, with the considerable advantages that oocytes offer over embryos for cryopreservation, has increased applications of oocyte cryopreservation in assisted reproduction techniques. A deep understanding of oocyte cryobiology, however, is lagging behind the forces propelling the clinical application of oocyte cryopreservation. We have drawn attention to this shortcoming by initiating a debate on whether a vitrified–warmed oocyte has the same characteristics as its fresh sibling. The answer to this question may explain why the oocyte cryopreservation success rate is as yet far from satisfactory and why cryopreserved oocytes should be treated differently from their fresh siblings. A fresh look at the characteristic features of oocytes after cryopreservation is the main scope of this review as a stimulus to further improvement of oocyte cryopreservation.

KEYWORDS: cryobiology, cryopreservation, oocyte

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Introduction

The cryobiology of reproductive cells dates back to an early study by Polge et al. (1949) on sperm freezing, which was followed 30 years later by cryopreservation of oocytes (Whittingham, 1977) and embryos (Whittingham, 1977). Although sperm and embryo freezing became routine soon after the first reports of their development, the earliest successful pregnancy us ing a previously frozen human oocyte was not reported until 1986 (Chen, 1986); however, this study did not generate much interest (until the 1990s) because it was initially considered that sperm and embryo cryopreservation fulfilled all the demands of fertility clinics and patients.

The increased activities of IVF clinics, however, made the shortcomings of embryo cryopreservation and the theoretical and practical advantages of oocyte versus embryo cryopreservation more evident. Indeed, oocyte cryopreservation increases the flexibility of assisted reproduction techniques by providing solutions for some of the critical religious, ethical, legal and clinical problems posed by embryo cryopreservation (Heng, 2007; Tucker et al., 2004; Van der Elst, 2003). Oocyte cryopreservation also allows patients to store oocytes whenever unforeseen conditions may occur, such as the inability of a partner to produce sperm, an adverse reaction of the patient to ovarian stimulation, or, for young women who face gonadotoxic treatments. Moreover, oocyte cryopreservation can be used as an alternative to embryo vitrification for donation to anovulatory women particularly in countries that have a ban on embryo vitrification, and eventually for young women wishing to delay child bearing (Heng, 2007; Koutlaki et al., 2006; Tucker et al., 2004; Van der Elst, 2003). These wide potential applications of oocyte cryopreservation have increased the pressure for addition of oocyte cryopreservation to the assisted reproduction technques repertoire.

In the intervening years, development of the new cryopreservation techniques of ultra-rapid cooling (vitrification) to replace the slow freezing method, thereby to improve cryosurvival, fertilization rates, the yield and quality of embryo development and pregnancy, led to renewed interest in oocyte cryopreservation technology (Kuwayama et al., 2005). As a consequence, the American Society for Reproductive Medicine (ASRM) has recently issued new guidelines on the controversial practice of oocyte cryopreservation, transferring it from an experimental procedure to a clinical practice. Although this will increase the application of oocyte cryopreservation, several important considerations should be addressed before oocyte cryopreservation can be added safely to assisted reproduction technique routine clinical practice. The aim of this review is to provide a fresh look at the exact status of oocytes after cryopreservation.

Is oocyte cryopreservation perfect?

Despite increasing reports of successful oocyte cryopreservation outcomes, both *in vitro* and after fertilization and transfer *in vivo*, the results of egg cryopresrvation are still mixed. Some studies on oocyte cryopreservation report relatively poor egg survival (Akin et al., 2007; Albani et al., 2008; Boldt et al., 2003, 2006; Borini et al., 2004, 2006; Chen et al., 2005; Cobo et al., 2001; Fabbri et al., 1998; Gook et al., 1994; Kazem et al., 2012; Levi Setti et al., 2006; Mandelbaum et al., 1988; Polak de Fried et al., 1998; Porcu et al., 2000; Yoon et al., 2003), in contrast to other reports of high survival of cryopreserved oocytes (Antinori et al., 2007; Cobo et al., 2008; Fosas et al., 2003; Katayama et al., 2003; Kohaya et al., 2013; Kuwayama et al., 2005; Li et al., 2005; Lucena et al., 2006; Parmegiani et al., 2011; Rienzi et al., 2010). Although the recent ASRM decision on oocyte cryopreservation designation as assisted reproduction technique mainstream was based on the results of four randomized clinical trials, the ASRM (www.asrm.org) has nonetheless declared that the success rate needs to be improved substantially. The variation among different oocyte cryopreservation reported outcomes may originate from many variables that affect the success rate, including factors related to oocyte donor (age, fertility and health status), stimulation protocol and IVF procedure (Cil and Seli, 2013), oocyte cryopreservation method (slow-freezing or vitrification: Boldt, 2011, Cobo and Diaz, 2011; Edgar and Gook, 2012, Kohaya et al., 2013), cryopreservation device (cryotops, cryoleaf, straw, cryotip, open pulled straw: Cil and Seli, 2013, Ledda and Naitana, 2007; Vajta et al., 2015), operator skill (Gualtieri et al., 2011), quality and maturation stage of oocytes used (Fabbri et al., 2001), and finally indications for oocyte cryopreservation (medical, nonmedical, or IVF-related reasons: Cil and Seli, 2013). Therefore, the actual state of oocyte cryopreservation can be reliably estimated only when these confounding effects are controlled using a randomized controlled trial study design (Cil and Seli, 2013). Another approach by which to evaluate the current status of oocyte cryopreservation is to compare the clinical results of oocyte cryopreservation with embryo cryopreservation. Embryo cryopreservation is now a well-integrated part of assisted reproduction techniques, and its efficiency is fairly comparable with, and even higher than, the transfer of fresh sibling embryos in survival and pregnancy rates (Zhu et al., 2011). If such a measure is used, the current efficiency of oocyte cryopreservation technique is low; small steps, but not giant leaps, have led to improving oocyte cryopreservation efficiency.

Why is oocyte cryopreservation less successful than embryo cryopreservation?

Mammalian oocytes are unique cells because of their developmental capacity to be fertilized and then to support early embryonic development (Hosseini et al., 2012; Sirard, 2012). This capacity derives from a maternal legacy of the myriad of transcripts, proteins and energetic substrates, and cytoplasmic organelles, which facilitate early mitotic divisions of the embryo until embryonic genome activation occurs (Sirard, 2012). This highly organized structure often incurs serious damage after cryopreservation (Asgari et al., 2011), and may explain why cryopreservation outcomes for such a large and complex cell differ from that of sperm or embryos. In fact, the volume of the mammalian oocyte is three to four orders time larger than that of the spermatozoa, thereby substantially decreasing the surface-to-volume ratio, and making them sensitive to chilling and highly susceptible to intracellular ice formation (Saragusty and Arav, 2011). In the early developing embryo, cleavage divisions occur without any net

increase in volume until the blastocyst stage (Aiken et al., 2004), leading to a significantly higher nucleus–cytoplasmic ratio of embryo blastomeres compared with the oocyte (Hosseini et al., 2013), and, correspondingly, oocytes are substantially more prone to cryo-damage than are embryos. Additionally, the number of blastomeres in an early stage embryo provides great flexibility to compensate for any detrimental effects of cryopreservation, because missed blastomeres can be replaced by the daughter cells of dividing intact ones. Oocytes contain one-half of the genetic material of the future individual, and so any damage to its chromatin structure may result in substantial deleterious defects in the developmental competence of the resulting embryo. Clear evidence shows that damage to the meiotic spindle can result in chromosomal abnormalities after thawing. Moreover, the permeability of oocyte plasma membrane to cryoprotective agents and water is low compared with embryo (Konc et al., 2014). An important issue that should be addressed in the comparison of vitrification efficiency between oocyte and embryo is the differential membrane permeability of cryoprotectant into or out of the oocyte and embryo. The cell membrane permeability of oocyte is an important factor in determining the conditions for cryopreservation (Jin et al., 2013). The permeating property differs not only with the stage of oocyte and embryo but also with the type of cryoprotectants (Jin et al., 2013). For instance, the permeability of mouse embryos generally increases as development proceeds to the compacted morula. At the one-cell stage, however, ethylene glycol is less permeating than propylene glycol, whereas in morulae, ethylene glycol is far more permeating than other cryoprotectants (Pedro et al., 2005). Moreover, it has been suggested that the exchange of water and cryoprotectants in expanded pig blastocysts occurs predominantly by facilitated diffusion but in oocytes predominantly by simple diffusion (Jin et al., 2013). This was related to the expression of aquaporin 3 mRNA, which was abundantly active in expanded blastocysts, but not in oocytes. The common consensus is that rapidly permeating agents are favoured for oocyte cryopreservation, because the exposure time before cooling can be shortened, and because osmotic swelling during removal of the cryoprotectant can be minimized. These lines of evidence (Saragusty and Arav, 2011) indicate why oocyte cryopreservation may not be as efficient as embryo cryopreservation.

Are mice relevant models for human oocyte vitrification?

The importance of animal models for the study and treatment of human diseases, combined with the scarcity of human oocytes for research studies, has led to the development of new technologies and optimization of existing oocyte cryopreservation data using animal models (Chang et al., 2011). Finding the appropriate model for the early stages of human embryo development, however, is still an open challenge. Rodents, therefore, particularly mice, were not only the first (Whittingham, 1977), but also remain the most popular (Demetrius, 2005) model species used in oocyte cryopreservation studies. Indeed, as much as 95% of all laboratory animals are rodents (Harkness et al., 2013). Mice are useful because of their small size and short generation times, and because breeding and keeping mice are comparatively simple and inexpensive. In addition, they have been widely used in research for decades, allowing researchers to build up a detailed understanding of mouse biology and genetics and to develop large numbers of tools and techniques to study them (Demetrius, 2005; Sylvestre *et al.*, 2013).

Mice, however, are not always reliable as preclinical models for human oocytes, and those of larger animals may be considered more suitable alternatives. For example, mice are multi-ovulatory animals, and the stage of zygote genome activation in mice is at the two-cell stage whereas human oocytes enter this stage two to three cell cycles later (Niakan and Eggan, 2013). Another concern is that different mouse strains have revealed different degrees of susceptibility to the vitrification process (Kohaya et al., 2013), which can confuse the evaluation of results between different studies. Moreover, comparative transcriptomic analyses of oocytes of different species have revealed that the bovine–human similarity is greater than mouse–human similarity (Sylvestre *et al.*, 2013). Also, a number of recent studies have demonstrated close similarities between human and sheep embryos in metabolism and key stages before and after implantation development (Barry and Anthony, 2008; Loi et al., 2011; McMillen, 2001), and correspondingly, it is suggested that the sheep is an relevant experimental model for human assisted reproduction techniques (Loi et al., 2011). Therefore, we have used sheep as an animal model to evaluate the fundamental aspects of oocyte cryobiology in human (Asgari et al., 2011, 2012a, 2012b; Hosseini et al., 2012, 2015a, 2015b).

In summary, other species including domestic animals (cattle, sheep, goats, dogs, pigs) and non-human primates may provide better models for human eggs than do mice. Importantly, the feasibility of obtaining an abattoir-derived source of oocytes in domestic animals and the established techniques of in-vitro oocyte maturation in these species may make them favourable models for future oocyte cryopreservation studies.

Is the cryopreserved oocyte the same oocyte as before cryopreservation?

Oocyte integrity is a key predictive indicator of oocyte quality that indicates its future developmental competence (Khalili *et al.*, 2012). The nature and magnitude of mechanical, chemical, osmotic and thermo-dynamical stresses often incurred during oocyte cryopreservation may be higher than the physiological capacity of the oocyte to withstand or ameliorate them. The effect of cryopreservation on the structural features of oocytes has been evaluated in several excellent studies (Bernard and Fuller, 1996; Fabbri et al., 2001; Potdar et al., 2014; Saragusty and Arav, 2011). All these studies provide evidence that the highly organized structure of fresh oocyte changes dramatically (at cellular, ultrastructural, molecular and developmental levels) after cryopreservation, and correspondingly, the cryopreserved oocyte has cellular characteristics that differ from those of the fresh oocyte.

In this sense, although a reasonable conclusion is that a cryopreserved oocyte should not be considered the same as an oocyte before cryopreservation, a critical question is: what does the cryopreserved oocyte look like? Before answering this question and and addressing the great list of changes that occur in the oocyte after cryopreservation, one may ask which

part of the changes occurring after oocyte cryopreservation have the major determining effect on subsequent development of the cryopreserved oocyte; this is addressed below.

Cryo-associated nuclear versus cytoplasmic damage: which is more important?

In a simplified pattern, cryo-associated damage to a second metaphase oocyte can be broadly divided between nuclear and cytoplasmic damage. Although the critical importance of both types of damage is well understood in the light of several recent studies (for review see Saragusty and Arav, 2011), an intriguing question is to determine the differential contributions of nucleus and cytoplasm to the poor quality of the vitrified oocyte. In an attempt to answer this question, we exchanged the karyoplast between vitrified and fresh oocytes to produce reconstituted oocytes containing either fresh–karyoplast–vitrified cytoplast (FK–VC) or vitrified–karyoplast–fresh–cytoplast (VK–FC) (Hosseini *et al.*, 2015a, 2015b) (**Figure 1**). Embryonic development in FK–VC reconstituted oocytes showed no improvement over control vitrification as assessed after either parthenogenetic activation or intracytoplasmic sperm injection (ICSI). The developmental competence of oocytes reconstituted by combining VK–FC, however, improved to rates comparable with control manipulations, and was significantly higher than FK–VC, VK–VC and control vitrification. These results point toward "cytoplasmic insufficiencies" as the main cause of poor competence of vitrified oocytes, which in turn highlights the essential need for technical modifications to current oocyte vitrification protocols. This model of karyoplast exchange may also have important implications for the cytoplasmic-rescue capacity of vitrified oocytes in the future repertories of assisted reproductive technologies. Why should the capacity of cleaved embryos derived from FK–VC reconstituted oocytes be significantly lower than VK–FC ones? The ability of the oocyte to develop into a viable embryo strongly depends on cellular and molecular aspects of nucleocytoplasmic maturation and the synthesis and storage of several components, i.e. RNAs, proteins, and energetic substrates, which are essential during the earliest stages of embryo development.

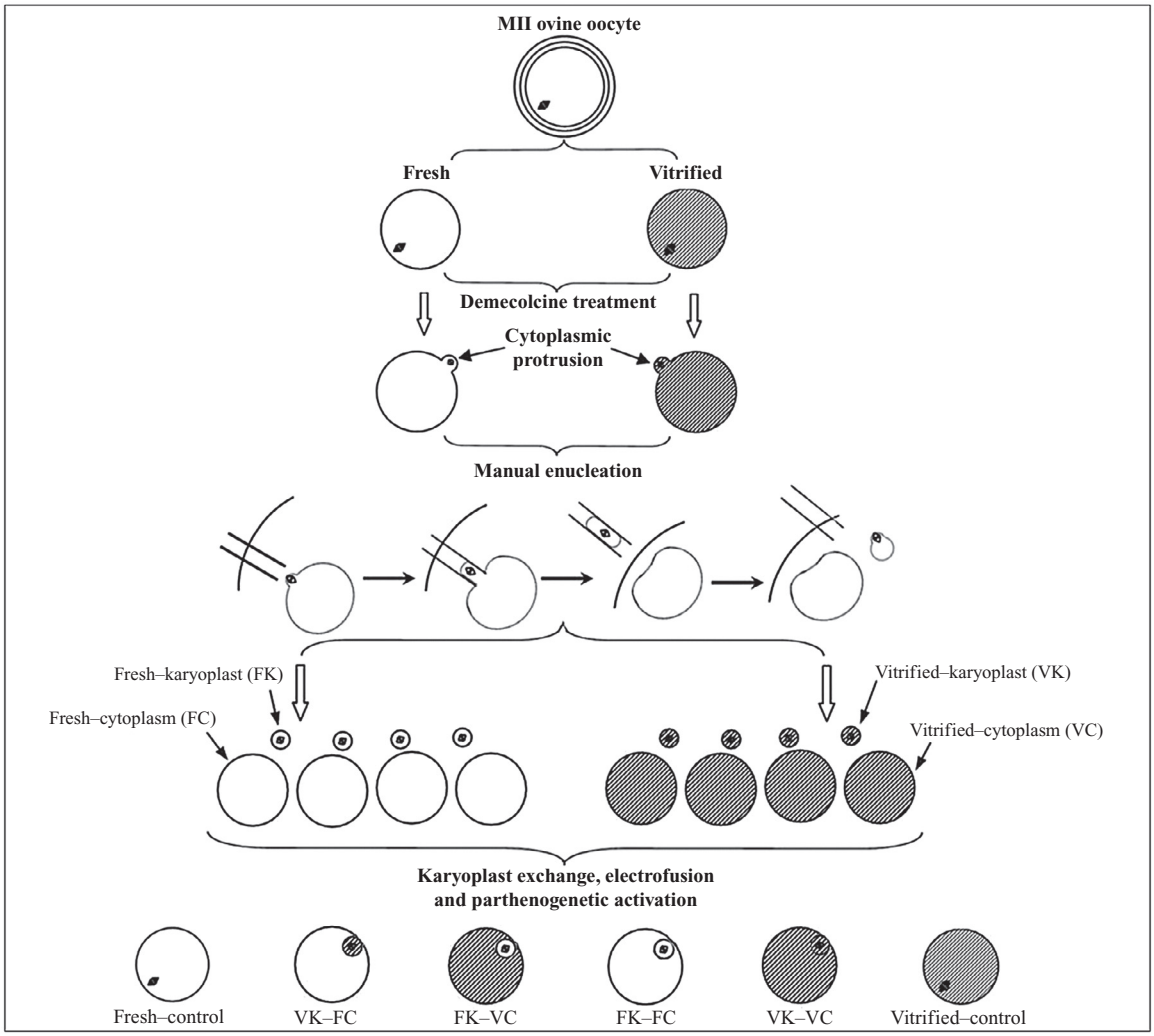


Figure 1 Experimental design for systematic comparison between cytoplasmic and nuclear damage incurred during investigation of matured oocytes. MII, second metaphase. Adapted from Hosseini et al. (2015a).

Cryopreservation may adversely affect many of these features and therefore the final capacity of cryopreserved oocytes is unexpectedly lower than fresh oocytes.

It is clear that DNA repair is a maternal trait. Therefore, the germinal vesicle oocyte is the source of transcripts of the main DNA repair pathways (base excision repair, direct reversal of damage, double strand break repair, mismatch repair and nucleotide excision repair), and plays a key role in the development of embryonic genome until the stage of embryonic genome activation (Jaroudi et al., 2009).

This powerful DNA repair machinery has chromatin assembly factors necessary for the maintenance of the genomic stability of the sperm nucleus or of transferred donor cell nuclei, as shown by recent studies of the development of embryos derived from oocytes injected with freeze-dried sperm containing a highly fragmented nucleus (Wakayama and Yanagimachi, 1998), or cytoplasts injected with dead cells containing denaturized chromatin structure owing to chemical agents (Loi et al., 2002) or freezing without cryoprotectants (Wakayama et al., 2008). One conclusion from these studies is the need for further attention to the cryobiology of the ooplasm to increase its survival during the cryopreservation process, a matter that is now discussed.

Does a post-warming interval favour oocyte survival and developmental competence?

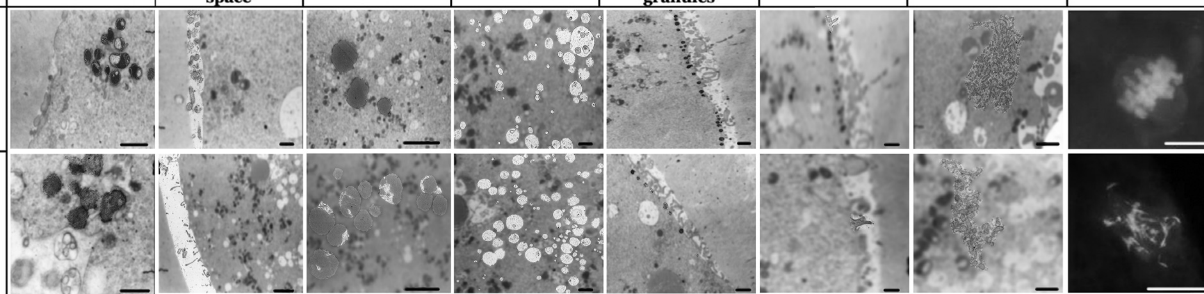
Broadly, the various types of damage occurring in cryopreserved oocytes can be divided into those that are reversible and those that are irreversible, based on the capacity of oocytes to recover (Agarwal, 2009; Asgari et al., 2012a, 2012b; Pickering et al., 1990; Wu et al., 2006). One issue for oocyte cryobiology is the requirement for a post-warming interval, a period of 1–3 h oocyte rest routinely implemented to allow the warmed-oocyte the chance to recover its integrity and reverse damage before a further treatment commences (Asgari et al., 2011; Hosseini et al., 2012). The specified time reflects the time needed for tubulin-repolymerization (Chen et al., 2001; Larman et al., 2007; Succu et al., 2013), although limited information exists about the oocyte's capability to restore other structural and ultrastructural changes occurring during cryopreservation (Hosseini et al., 2012).

In a series of studies focused on cellular, molecular, ultrastructural and developmental characteristics of vitrified oocytes assessed either immediately or 2–3 h after warming recovery time was found to be beneficial for microtubule repolymerization and spindle recovery, and hence chromosome rearrangement; however, it was found that elements of oocytes that are intimately involved in second metaphase arrest may become modified, such that vitrified-warmed oocytes resume meiosis and progress toward the second telephase (Asgari et al., 2011, 2012a, 2012b; Hosseini et al., 2012, 2013). Spontaneous and parthenogenetic activation of cryopreserved oocytes has been reported in a number of studies (Bernard and Fuller, 1996; Bogliolo et al., 2007; Gook and Edgar, 2007; Larman et al., 2007; Succu et al., 2013; Tian et al., 2007). Indeed, all these studies provided evidence that cryo-associated alterations in the central kinase of meiosis inhibition, maturation promoting factor, leads to alterations in the microfilaments overlying the meiotic spindle and hence activation of the cryopreserved oocyte. Studies on vitrified ovine oocytes indicated no significant increase in the survival rate after inclusion of a 2–3 h post-warming interval, whereas most of the rested oocytes resumed meiosis and progressed towards second anaphase stage within 2 h in the absence of any additional stimulus. These oocytes could also develop a female pronucleus and even initiate embryonic division before arresting permanently at the stage of zygote genome activation (Asgari et al., 2011; Bogliolo et al., 2007; Gook and Edgar, 2007; Hosseini et al., 2012; Larman et al., 2007; Succu et al., 2013; Tian et al., 2007).

Considering the critical importance of cell cycle synchrony between male and female pronuclei during IVF and ICSI for correct ploidy and developmental competence of the developing embryo, possible detrimental effects of a post-warming interval should be considered in more detail before incorporating it into routine protocol of human oocyte cryopreservation. Interestingly, when polarized light microscopy was used to measure meiotic spindle retardance, Larman et al. (2007) demonstrated that the main reason for microtubule depolymerization during oocyte cryopreservation is exposure to the temperatures below 37°C but not to the cryoprotectants. Therefore, the treatment of vitrified oocytes can proceed without having to await spindle recovery.

What does the cryopreserved oocyte look like?

Recent studies on the characteristics of cryopreserved oocytes have provided a huge database on oocyte cryobiology (Bernard and Fuller, 1996; Fabbri et al., 2001; Potdar et al., 2014; Saragusty and Arav, 2011). The common consensus is that oocyte cryopreservation does not significantly impair the general microarchitecture of the oocyte. Under normal light microscopy, the cryopreserved oocyte is almost normal in shape, size and dimensions (Nottola et al., 2009). One point highlighted by almost all ultrastructural studies, however, is the marked differences between the ultrastructural characteristics of cryopreserved and fresh oocytes. The provision of a definitive picture of a cryopreserved oocyte would help us to create the development of an optimized oocyte-tailored cryopreservation protocol. Therefore, in a series of studies, we attempted a comparison of the cellular, molecular, ultrastructural and developmental characteristics of vitrified, young, aged, activated, and fertilized matured oocytes (Asgari et al., 2011, 2012a, 2012b; Hosseini et al., 2012, 2013). Much evidence obtained argues that the process of vitrification leaves second metaphase oocytes with multifaceted features that differ from the initial intact second metaphase oocytes, and resemble more those of unvitrified oocytes subjected to parthenogenetic activation or in-vitro ageing (**Figure 2**). Healthy mitochondria are rounded or oval in shape with a few peripheral or transverse cristae. Dividing mitochondria may also be found as dumbbell-shaped structures within the cytoplasm (Asgari et al., 2011; Wahid et al., 2012; Wu et al., 2006). Damaged mitochondria lose their rounded shape and are swollen in size with reduced density and alteration of the cristae. Voluminous aggregates between mitochondria and smooth endoplasmic reticulum are seen (Wahid et al., 2012; Wu et al., 2006). The perivitelline space and microvilli are closely associated structures, and cryopreservation shocks (temperature and osmotic shocks) cause alterations



	Mitochondria	Perivitelline space	Lipid droplets	Vacuoles	Cortical granules	Microvilli	Endoplasmic reticulum	Spindle-chromosomes
Normal Features								
Abnormal features								
Vitrified oocytes	Wu *et al*., 2006; Wahid *et al.*, 2012;Chaves *et al*., 2014; Asgari *et al*., 2011; Hosseini *et al*., 2012; Succu *et al*., 2007; Ebrahimi *et al*., 2012; Rho *et al*., 2002; Turathum *et al*., 2010; Bernard and Fuller, 1996.	Wahid *et al*., 2012; Chaves *et al*., 2014; Asgari *et al*., 2011; Hosseini *et al*., 2012; Succu *et al*., 2007; Ebrahimi *et al*., 2012; Turathum *et al*., 2010; Bernard and Fuller, 1996.	Wu *et al*., 2006; Wahid *et al*., 2012; Chaves *et al*., 2014; Asgari *et al*., 2011; Hosseini *et al*., 2012; Succu *et al*., 2007; Ebrahimi *et al*., 2012; Modina *et al*., 2004; Turathum *et al*., 2010; Bernard and Fuller, 1996.	Wu *et al*., 2006; Wahid *et al*., 2012; Chaves *et al*., 2014; Asgari *et al*., 2011; Hosseini *et al*., 2012; Succu *et al*., 2007; Ebrahimi *et al*., 2012; Modina *et al*., 2004; Turathum *et al*., 2010; Bernard and Fuller, 1996.	Wu *et al*., 2006; Wahid *et al*., 2012; Chaves *et al*., 2014; Asgari *et al*., 2011; Hosseini *et al*., 2012; Succu *et al*., 2007; Ebrahimi *et al*., 2012; Modina *et al*., 2004; Turathum *et al*., 2010; Bernard and Fuller, 1996.	Wu *et al*., 2006; Wahid *et al*., 2012; Chaves *et al*., 2014; Asgari *et al*., 2011; Hosseini *et al*., 2012; Succu *et al*., 2007; Ebrahimi *et al*., 2012; Modina *et al*., 2004; Turathum *et al*., 2010; Bernard and Fuller, 1996.	Wu *et al*., 2006; Wahid *et al*., 2012; Chaves *et al*., 2014; Asgari *et al*., 2011; Hosseini *et al*., 2012; Succu *et al*., 2007; Ebrahimi *et al*., 2012; Modina *et al*., 2004; Turathum *et al*., 2010; Bernard and Fuller, 1996.	Wu *et al*., 2006; Wahid *et al*., 2012; Chaves *et al*., 2014; Asgari *et al*., 2011; Hosseini *et al*., 2012; Succu *et al*., 2007; Ebrahimi *et al*., 2012; Modina *et al*., 2004; Rho *et al*., 2002; Turathum et *al*., 2010; Tamura *et al*., 2013; Bernard and Fuller, 1996.
Activated oocytes	Asgari *et al*., 2011; Hosseini *et al*., 2012; Hao *et al*., 2009.	Asgari *et al*., 2011; Hosseini *et al*., 2012.	Asgari *et al*., 2011; Hosseini *et al*., 2012; Hao *et al*., 2009.	Asgari *et al*., 2011; Hosseini *et al*., 2012.	Wang *et al*., 1997; Asgari *et al*., 2011; Hosseini *et al*., 2012.	Asgari *et al*., 2011; Hosseini *et al*., 2012.	Asgari *et al*., 2011; Hosseini *et al*., 2012.	Asgari *et al*., 2011; Hosseini *et al*., 2012; Kim *et al*., 1996.
Aged oocytes	Díaz and Esponda, 2004; Hosseini *et al*., 2012; Hao *et al*., 2009; Maio *et al*., 2009.	Hosseini *et al*., 2012; Maio *et al*., 2009.	Hosseini *et al*. 2012; Hao *et al*., 2009; Maio *et al*., 2009.	Hosseini *et al*., 2012; Maio *et al*., 2009.	Díaz and Esponda, 2004; Hosseini *et al*., 2012; Ducibella *et al*., 1990; Szollosi, 1971; Gulyas, 1979; Maio *et al*., 2009.	Díaz and Esponda, 2004; Hosseini *et al*., 2012; Maio *et al*., 2009.	Hosseini *et al*., 2012; Maio *et al*., 2009.	Hosseini *et al*., 2012; Szollosi, 1971; Mailhes *et al*., 1998; Maio *et al*., 2009; Kim *et al*., 1996.

Figure 2 Outline of the ultrastructural characteristics of vitrified oocytes, which are similar to those seen in aged and activated oocytes. The images in upper row denote normal features of oocyte ultrastructure and the lower row denote the abnormal features of the same structures as frequently observed in vitrified, activated and aged oocytes. Bars in all figures represent 5 μm.

in these structures simultaneously; the perivitelline space enlarges in size and the number and the length of microvilli projections into the perivitelline space reduces after cryopreservation (Asgari et al., 2011; Bernard and Fuller, 1996). Lipid droplets are typically round in fresh second metaphase oocytes but became smaller and less electron-lucent after cryopreservation, and their membranes are broken and mitochondria are infiltrated inside lipid droplets (Hosseini et al., 2012; Wu et al., 2006). A slight degree of cytoplasmic vacuolization may be normal in immature oocytes. In contrast, vacuolization in cryopreserved second metaphase oocytes may be considered as a non-specific response of the oocyte caused by swelling and coalescence of isolated smooth endoplasmic reticulum (Wu et al., 2006). Cortical granules in fresh second metaphase oocytes are observed as abundant spheres, which are aligned at the periphery of oocyte, under the oolemma. After cryopreservation, cortical granules form a discontinuous layer and their amount and density is abnormally reduced, owing to massive exocytosis into the perivitelline space leading to hardening of the inner face of the zona and polyspermy (Asgari et al., 2011; Hosseini et al., 2012). In fresh oocytes, aggregates of anastomosing tubuli of smooth endoplasmic reticulum surrounded by mitochondria are the most abundant oocyte organelle. After cryopreservation, no drastic changes are observed in the ultrastructure of these aggregates but they may be partially replaced by swelling smooth endoplasmic reticulum associated with swollen mitochondria. A characteristic feature of second metaphase oocytes is the presence of metaphase chromosome carefully aligned on a well-developped spindle. Cryopreserved oocytes show different degrees of abnormality in both structures with lagging chromosomes scattered within the cytoplasm with abnormal and ruptured spindles (Asgari et al., 2011; Hosseini et al., 2012). Therefore, vitrified oocytes revealed a time-dependent decline in quality, and notably, the speed of this decline in quality was higher in vitrified-young oocytes, indicating that once warmed, the vitrified oocytes should be used for the next treatment(s) as soon as possible (Asgari et al., 2012a, 2012b; Hosseini et al., 2012).

We also observed that the sensitivity of parthenogenetic activation of vitrified oocytes was similar to that of in-vitro

aged oocytes, both types of oocytes requiring a higher amplitude and longer duration of calcium ionophore treatment to provoke the intracellular calcium oscillations characterizing oocyte activation (Asgari et al., 2012a, 2012b; Hosseini et al., 2012). Importantly, step-wise assessment of oocyte responses to the different steps of the vitrification-warming cycle showed that the first stage of dehydration, at the beginning of vitrification process, when oocytes are routinely exposed to cryoprotectants, may be the more challenging stage and is more likely to render the oocyte prone to damage subsequently (Asgari et al., 2012a, 2012b; Hosseini et al., 2012, 2015a). In one measure, cryoprotectant damage, whether toxic or osmotic, was of negligible importance compared with cryoshocks (chilling injury) for the integrity of the vitrified oocytes. In agreement, some studies have successfully used vitrification solutions containing high concentrations of propylene glycol (Arav et al., 1993) or ethylene glycol (Mochida et al., 2013). Using sheep as a model animal, we observed that the inferior quality of vitrified oocytes is more pronounced when the oocytes have to remodel the sperm (whether introduced by IVF or by ICSI), compared with their potential to support parthenogenetic activation and somatic cloning, after which development remained almost unaltered (Hosseini *et al.*, 2015a, 2015b). Therefore, one may argue that the cytoplasm of the vitrified oocyte has the necessary components to support in-vitro embryonic development of the maternal, or even adult somatic cell, chromosomes, but fails to do so with sperm chromosomes.

In one assumption, a vitrified-warmed oocyte seems to have cellular characteristics resembling unvitrified-activated or unvitrified, in-vitro aged oocytes rather than the initial intact one (**Figure 2**). This information may provide a more useful image of oocyte cryobiology for future improvement of oocyte cryopreservation techniques. Nevertheless, considering different success rates of oocyte vitrification in human and ovine species, as well as the different variations in oocyte maturation and vitrification protocols, one must consider that this conclusion may be limited to this animal model, in the case of in-vitro matured oocytes, and under this vitrification protocol.

Clinical implication of nuclear DNA-transplantation to rescue cytoplasmic insufficiencies of vitrified matured oocytes

In February 2015, the UK parliament approved the licensing of nuclear DNA or cytoplasmic transplantation by the Human Fertilization and Embryology Authority through a controversial amendment to the 2008 Human Fertilization and Embryology Act as an assisted reproduction technique service to rescue the mitochondrial insufficiency of oocytes in genetically affected patients. Cytoplasmic transplantation is not a new technique, and a variety of studies have used it to answer fundamental questions about the molecular mechanisms of nucleocytoplasmic interactions and to overcome ooplasmic deficiencies. Cytoplasmic transfer in humans was successfully used for the birth of over 30 babies until 2001 (Barritt et al., 2001). At that time, the main philosophy behind these studies led by Jacques Cohen was to restore the quality of suspect cytoplasm (including mitochondria) of the oocytes of the patients (Barritt et al., 2001). Although mitochondria are an important determining factor of oocyte quality, the list of well-established defects that occur during oocyte vitrification encouraged us to investigate whether cytoplasmic transfer between fresh and vitrified oocytes may also help to rescue the insufficiencies of the cytoplasm of the vitrified oocyte as a whole. Our preliminary studies in an ovine model (unpublished data), using a modified technique of karyoplasm exchange between fresh and vitrified zona-intact oocytes (**Figure 3**) combined with ICSI, resulted in a great improvement in cleavage and embryonic development of those vitrified oocytes that received fresh cytoplasm compared with intact vitrified oocytes. Although the results are too preliminary to reach a final conclusion, further studies may suggest that this model of karyoplast exchange offers a straightforward approach to rescue the cytoplasmic insufficiencies of vitrified oocytes.

Epigenetic consequences of oocyte cryopreservation: a call for investigation

Despite the indispensable importance of oocyte cryopreservation in the current repertoire of human assisted reproductive techniques, an emerging body of recent studies provides evidence suggesting that assisted reproduction techniques may significantly affect the epigenetic status of the oocyte and developing embryo, with potential implications for the success rate and safety of assisted reproduction techniques (Batcheller et al., 2011; Eroglu and Layman, 2012; Nejat and Buyuk, 2012; Niemitz and Feinberg, 2004). During recent studies, we observed that the epigenetic signature of the oocyte and early embryo is sensitive to cryopreservation, the H3K9 acetylation being increased significantly after warming (Bakhtari et al., 2014; Bonakdar et al., 2015), which is in agreement with other studies (Spinaci et al., 2012; Yan et al., 2010). Developmentally, the maternal epigenome undergoes massive changes after fertilization, including gradual demethylation, or more precisely, increased hydroxymethylation, which is followed by another round of epigenetic changes during de-novo methylation and cell specification (Jafari et al., 2011; McGraw et al., 2007). With these two critical stages of epigenomic regulation, it may be that the epigenetic signature of the oocyte does not necessarily predict the epigenome pattern of the future embryos. Further studies are needed to understand clearly the extent to which mediated changes to the oocyte epigenome caused by assisted reproduction techniques may be corrected during subsequent stages of embryo development. For example, in a recent study (Bonakdar et al., 2015), we systemically compared in mice the long-lasting effects of superovulation, vitrification, in-vitro culture and embryo transfer on the expression of epigenetic modulators, imprinted genes and pluripotency markers in blastocysts and E9.5 concepti. The results showed that, among the assisted reproductive techniques assessed, superovulation adversely affected fetal growth during post-implantation development more than in-vitro culture, vitrification and embryo transfer. This evidence suggests that the physiological post-implantation environment can attenuate some of the compromising effects of vitrification often incurred during oocyte and embryo freezing.

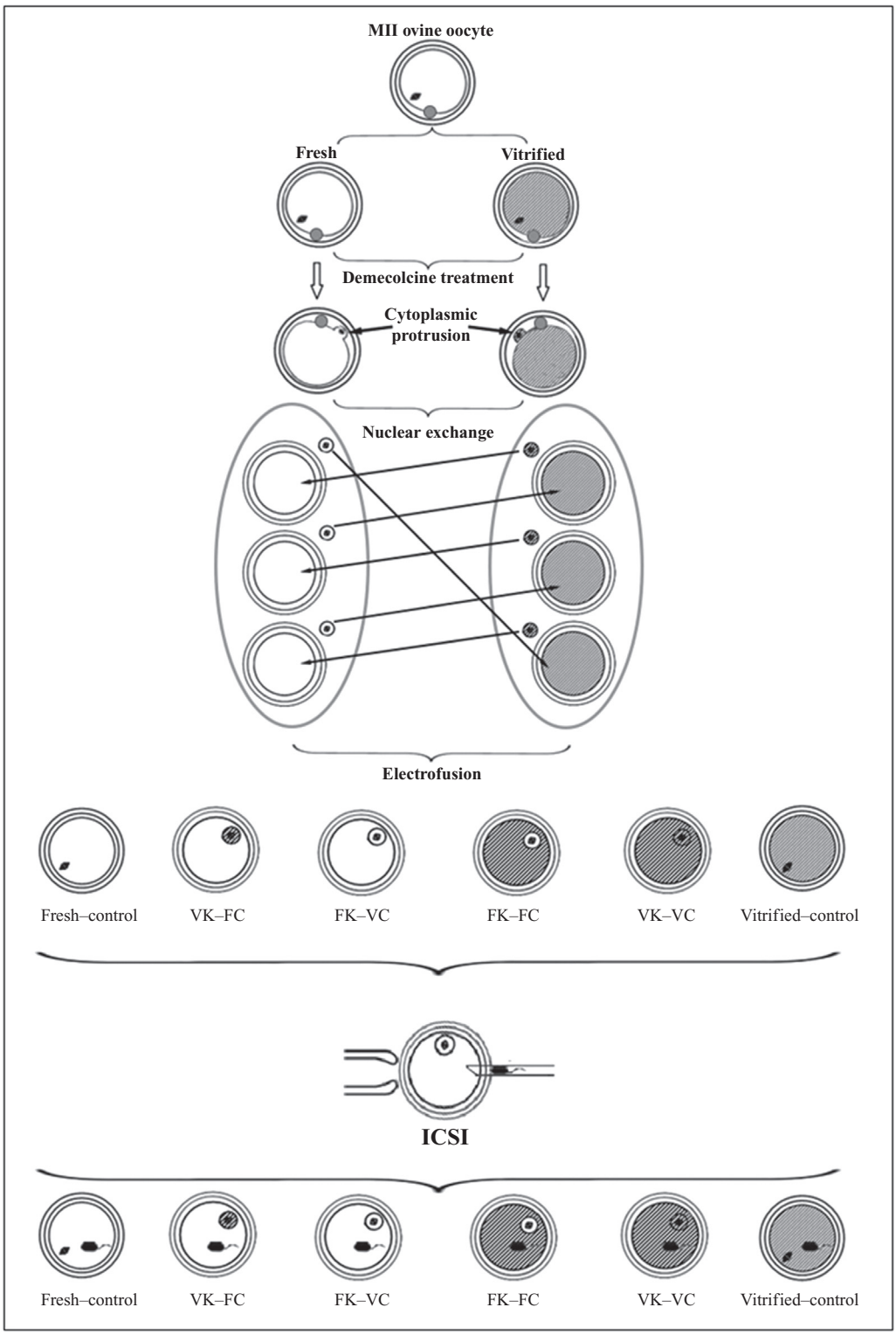


Figure 3 Graphical hypothesis: Potential implication of cytoplasmic transfer to rescue cytoplasmic insufficiency of vitrified oocytes. If cytoplasmic damage is the main cause of poor developmental competence of vitrified oocytes, then cytoplasmic transfer from fresh oocytes into vitrified oocytes would improve developmental competence of vitrified oocytes after intracytoplasmic sperm injection.

Conclusions

The ability to store oocytes confers significant benefits for human medicine, agriculture and wildlife conservation. Overt advantages of oocyte versus embryo cryopreservation, along with the validation of oocyte cryopreservation in the current repertoire of assisted reproduction technqiue services achieved by introduction of the vitrification technique, have

increased its use in assisted reproduction technique clinics. Despite this, the current success rate of oocyte cryopreservation is far from satisfactory, and increasing the efficiency of oocyte cryopreservation is a difficult but realistic objective. Data presented in this review provide evidence that a vitrified-warmed oocyte should not be assessed, conceptualized, and therefore treated, as being identical to its sibling fresh one. In one measure, a vitrified-warmed sheep second metaphase oocyte has characteristics similar to an in-vitro aged and parthenogentically activated oocyte. Such a consideration of a vitrified–warmed oocyte may provide a platform for better understanding of mature oocyte cryobiology, which has important implications for development of an optimized oocyte-tailored cryopreservation protocol.

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