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Bio-inspired Solute Enables Preservation of Human Oocytes using Minimum Volume Vitrification

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Abstract

The ability to cryopreserve human oocytes has significant potential for fertility preservation. Current cryopreservation methods still suffer from the use of conventional cryoprotectants, such as dimethyl sulfoxide (DMSO), causing loss of viability and function. Such injuries result from the toxicity and high concentration of cryoprotectants as well as mechanical damage of cells due to ice crystal formation during the cooling and rewarming processes. Here, we report preservation of human oocytes following vitrification using an innovative bio-inspired cryoprotectant integrated with a minimum volume vitrification approach. The results demonstrate that the recovered human oocytes maintained viability following vitrification and rewarming. Moreover, when this approach was used to vitrify mouse oocytes, the recovered oocytes preserved their viability and function following vitrification and rewarming. This bio-inspired approach substitutes DMSO, a well-known toxic cryoprotectant, with ectoine, a non-toxic naturally occurring solute. The bio-inspired vitrification approach has potential to improve fertility preservation for women undergoing cancer treatment and endangered mammal species.

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Author's roles

J.K.C., and U.D., developed the idea; J.K.C., R.E., R.M.A., and U.D designed the experimental approach; J.K.C., and N.N., performed the experiments; J.K.C., R.E., E.G., R.M.A., and U.D., analyzed the data; J.K.C., R.E., E.G., R.M.A., and U.D., wrote the manuscript.

Conflict of interest

Dr. U Demirci is a founder of, and has an equity interest in: (i) DxNow Inc., a company that is developing microfluidic and imaging technologies, (ii) Koek Biotech, a company that is developing microfluidic IVF technologies for clinical solutions, and (iii) LEVITAS Inc., a company that develops biotechnology tools for genomic analysis in cancer. U.D.'s interests were viewed and managed in accordance with the conflict of interest policies.

Keywords

Bio-inspired Material; Naturally inspired; Ectoine; Low level of cryoprotectants (CPAs); Human Oocyte; Vitrification

Introduction

Oocyte cryopreservation is a rapidly growing technology to preserve female fertility (Asghar, *et al.*, 2014, Domingo and Garcia-Velasco, 2016, Zhang, *et al.*, 2011). It has been used as an alternative to embryo freezing providing women with additional options for fertility preservation and deferring child bearing (Goldman and Grifo, 2016, Konc, *et al.*, 2014, Tao and Del Valle, 2008). Currently, two cryopreservation methods are used for long-term storage of oocytes: (i) slow freezing and (ii) vitrification (Asghar, *et al.*, 2014, Meryman, 2007, Zhang, *et al.*, 2011). The slow freezing method presents low toxicity for oocytes due to the use of low concentration of cryoprotective agents (CPAs; ~1–2M). The main challenge in slow freezing is the intracellular ice formation (IIF) during oocyte cryopreservation, as it can lead to oocyte damage and developmental arrest (Asghar, *et al.*, 2014, Saragusty and Arav, 2011, Zhang, *et al.*, 2012). On the other hand, vitrification, in which high cooling rates in combination with high concentration of CPAs (up to ~8M) are used, does not show IIF (Edgar and Gook, 2012, Martínez-Burgos, *et al.*, 2011). However, in vitrification, CPAs have become increasingly toxic for oocytes due to the need for high CPA concentrations to achieve vitrification. Recently, minimum volume vitrification methods have emerged to reduce the cryo-damage associated with conventional vitrification methods (Saragusty and Arav, 2011, Zhang, *et al.*, 2011). For example, glass-based microcapillary (e.g., quartz microcapillary (QMC)) techniques have shown potential in cryopreserving cells due to their high thermal conductivity (~130,000–250,000 °C/min) (Arav, *et al.*, 2002, Choi, *et al.*, 2015a, Risco, *et al.*, 2007). It is thus of great need to develop methods that combine advantages of slow freezing (low toxicity) and vitrification (no ice formation) for oocyte cryopreservation.

Conventional cryopreservation methods utilize two main types of CPAs: (i) permeating CPAs (e.g., dimethyl sulfoxide (DMSO), 1,2-propanediol, and ethylene glycol (EG)), which infiltrate through the cell membrane, and protect the intracellular structures of cells; (ii) permeating CPAs (e.g., poly (ethylene glycol), poly (vinyl pyrrolidone), hydroxyethyl starch, and trehalose), which cannot infiltrate through the cell membrane due to their large molecular weight. These CPAs have shown a complimentary protective effect by stabilizing the outer layer of cell membrane and inhibiting the extracellular ice formation (Asghar, *et al.*, 2014, Jang, *et al.*, 2017). Existing oocyte cryopreservation methods use toxic cryoprotectants such as DMSO, which is associated with toxic effects on cells (e.g., cytotoxicity) (Asghar, *et al.*, 2014). The deleterious effects posed due to exposure of cells to conventional CPAs can be avoided by using non-toxic naturally occurring cryoprotectants. Recently, the ability to protect cells (e.g., stem cells and red blood cells) from cryo-damage has been reported using bio-inspired cryoprotectants, such as ectoine (El Assal, *et al.*, 2014, Grein, *et al.*, 2010, Guven and Demirci, 2012), trehalose (Eroglu, *et al.*, 2000, Zhang, *et al.*, 2016), and anti-freeze proteins (Carpenter and Hansen, 1992, Mitchell, *et al.*, 2015). Ectoine is a

biocompatible osmolyte discovered in the halophilic bacterium living in extreme environments (*e.g.*, high salt concentrations and very low temperatures)(Buommino, *et al.*, 2005, Roberts, 2005). The use of such bio-inspired non-toxic materials can eliminate or reduce the deleterious effects observed with conventional CPAs, resulting in improving the viability and functionality of cryopreserved oocytes.

In this study, we report the ability to preserve human oocytes following minimum volume vitrification using ectoine as a naturally-inspired cryoprotectant. We first validated this bio-inspired approach using mouse oocytes. The recovered mouse oocytes were evaluated for viability and function demonstrated via parthenogenetic activation after vitrification and rewarming. Subsequently, we applied this approach to cryopreserve human oocytes, and the viability of recovered cells was evaluated after vitrification and rewarming.

Results and Discussion

To achieve minimum volume vitrification, we utilized quartz microcapillary (QMC) to encapsulate oocytes (Figure 1a). In this method, a QMC with a wall thickness of 10 μm was used for oocyte vitrification. Oocytes were loaded with the ectoine-based bio-inspired CPA solution (*i.e.*, 0.7M ectoine, 1.4M ethylene glycol (EG), and 1.3M trehalose) into QMC and plunged into liquid nitrogen. The optimal concentration of ectoine in the CPA solution was determined by set of minimum volume vitrification experiments using QMC (Supplementary Figure 1). The oocytes were then rewarmed in PBS with 0.2M trehalose at 37°C (Figure 1b). It is worth mentioning that utilizing QMC does not impede rapid rewarming as it has been reported in the literature(Javed, *et al.*, 2011, Lee, *et al.*, 2010, Risco, *et al.*, 2007). We compared our results with DMSO as the standard CPA (*i.e.*, 1.1M DMSO, 1.4M EG, and 1.3M trehalose). In this study, we focused on replacing DMSO, a well known toxic CPA, with ectoine, a non-toxic bio-inspired CPA; therefore, we reduced the variable by keeping EG and trehalose concentrations constant (*i.e.*, 1.4M and 1.3M, respectively) in both cocktail solutions (*i.e.*, DMSO- and ectoine-based CPAs) and using the same method (*i.e.*, QMC) for the experimental design.

Various vitrification devices, including conventional plastic straw have been used for vitrification of many cells including oocytes(Asghar, *et al.*, 2014, Berthelot, *et al.*, 2000, Chung, *et al.*, 2000, Hyttel, *et al.*, 2000, Nöthling and Shuttleworth, 2005, Topal-Celikkan, *et al.*, 2015). However, due to their low cooling rates (~ 1000 °C/min), these methods require high CPA concentrations (~ 8 M), which further subject the cells to high levels of toxicity. Newly developed methods such as cryo-top, cryo-tip, and QMC have shown a significant advantage in increasing cooling rates (< 12000 °C/min)(Zhang, *et al.*, 2011). Therefore, we here employed the QMC method, which is a highly conductive material with a thin-walled microcapillary to provide ultra-high cooling rate(Lee, *et al.*, 2010). Such high cooling rates were earlier shown to reduce the need of high levels of CPA to achieve vitrification. In addition, closed vitrification systems such as QMC and cryo-top have advantages over conventional open vitrification systems. For example, there is no risk of viral, bacterial, and fungal contaminations when using closed system methods compared to open system methods, which allow the oocytes to be in direct contact with liquid nitrogen with a possible risk of contamination (Bielanski, 2012, Bielanski and Vajta, 2009, Kuwayama, *et al.*, 2005).

This minimal volume vitrification method (*i.e.*, droplet in a QMC) allows elimination of toxic CPA (*e.g.*, DMSO) with biocompatible bio-inspired solution based on ectoine. Ectoine is a biocompatible naturally occurring solute present in extremophile, which has the ability to survive in extreme environments such as cold, hot, and salty conditions (Lippert and Galinski, 1992, Roberts, 2005). Ectoine is a low molecular weight permeating CPA, and can accumulate intracellularly at high concentrations without interfering with normal cellular activities (Bownik and Stępniewska, 2016). Ectoine has been synthesized and used for decades in various biomedical applications (*e.g.*, skin protection, anti-inflammatory drugs, and neurodegenerative treatment) (Bownik and Stępniewska, 2016, Graf, *et al.*, 2008, Heinrich, *et al.*, 2007, Kanapathipillai, *et al.*, 2005, Marini, *et al.*, 2013, Sydlík, *et al.*, 2009). Although, the protective mechanism of action has not been thoroughly investigated, it is most likely associated with the preferential exclusion model of ectoine (Roberts, 2005). The exclusion stabilizes biomolecules and the lipid mono- and bi-layers of cell membrane, which helps cells to withstand extreme temperatures or osmotic stress (Harishchandra, *et al.*, 2010). The ectoine solutes are excluded from the direct interaction with hydrated shell of protein, preventing the denaturation of cellular proteins (Eiberweiser, *et al.*, 2015). Due to its protective characteristics and non-toxic nature, ectoine-containing CPA solutions are recently being utilized for cryopreservation of various cell types such as red blood, mononuclear, stem, and endothelial cells (Bissoyi and Pramanik, 2013, El Assal, *et al.*, 2014, Grein, *et al.*, 2010, Sun, *et al.*, 2012). In addition, other DMSO-free methods have been developed to cryopreserve oocytes. For example, a S3 vitrification system, which uses a series of three DMSO-free vitrification solutions (*i.e.*, EG and glycerol) and a 0.25cc sterile straw (as a cooling tool), has shown improved blastocyst vitrification outcomes (Stachecki and Cohen, 2009, Wiemer and Stachecki, 2015). By lowering the CPA concentration, and using stepwise loading/unloading processes, we also have reduced the osmotic stress associated with loading/unloading steps. The loading and unloading steps are less stressful on cells compared to the high osmotic stress/injury typically observed with the existing methods that are generally used (*i.e.*, high concentration CPAs up to ~8M) for oocyte and embryo vitrification. It is well-known that osmotic stress is critical during loading and unloading of CPAs. Reducing osmotic shock to cells has been addressed in the literature by stepwise loading and unloading of CPAs or by using microfluidic gradual loading/unloading methods, some of which were earlier reported (Song, *et al.*, 2009, Zhang, *et al.*, 2012).

To validate our method before testing it on human oocytes, we first used mouse oocytes and evaluated their viability following ectoine-based vitrification and rewarming (Figure 2; Figure 1c). A live/dead assay was used to stain 30 mouse metaphase II (MII) oocytes per group in the ectoine-based (sample) and DMSO-based (control) groups (Figure 2a-h). The viability results demonstrated no significant difference between sample (93%; 28/30) and control group (93%; 28/30) (Figure 2i). Figure 2 shows the morphology of vitrified live and dead mouse oocytes using phase and fluorescence microscopy.

To assess the functionality of cryopreserved mouse oocytes, we performed embryonic development (from two cells to blastocyst (32 cells)) (Figure 3; Figure 1d). This analysis was used to confirm the developmental ability of recovered mouse oocytes via parthenogenetic activation, which mimics oocyte fertilization (Figure 3a). A total number of 41, 40, and 40 mouse MII oocytes, were used for embryonic development in (i) no

Materials and Methods

Animals and materials

Female B6CBAF1 mice were purchased from Jackson Laboratory and maintained on a 16–8 h light–dark cycle. All procedures for animal use were approved by the Institutional Animal Care and Use Committee (IACUC) at Stanford University and every effort was made to minimize animal suffering. All Materials were purchased from Sigma unless otherwise stated.

Human oocytes

61 discarded, unfertilized, and anonymous human oocytes (MII, MI, and GV) were obtained from consenting patients already undergoing IVF cycle(s) at Brigham and Women's Hospital (BWH). They were approved by institutional review board (IRB; protocol # 30309 (Stanford University), 2012P000613 (BWH)).

Retrieval of mouse oocytes by superovulation

The 6–8 week old B6CBAF1 female mice were superovulated with 5 IU of pregnant mare serum gonadotropin (PMSG) and 5 IU of human chorionic gonadotropin (hCG) administered by intraperitoneal (i.p.) injections 48 h apart. Fifteen hours after the hCG injection, the oviducts were collected. The cumulus–oocyte complexes (COCs) were released from the ampullary region of each oviduct by puncturing the oviduct with a 28-gauge needle and collected by flushing the oviduct with 1 ml flushing hold medium (FHM) supplemented with 4 mg/ml bovine serum albumin (BSA). To obtain clean oocytes, the COCs were incubated in FHM medium containing 200 IU/ml hyaluronidase at 37°C for up to 3 min to detach cumulus cells and further washed three times using fresh FHM medium. MII oocytes with a first polar body were cultured in calcium-free Potassium Simplex Optimized Medium (KSOM; Millipore) at 37°C and 5% CO₂ till before further experimental use.

Vitrification of mouse and human oocytes

Mouse oocyte vitrification—We loaded cryoprotective agent (CPA) into mouse oocytes (at the MII stage) in two steps. This was done by first incubating the oocytes with a pre-equilibrium solution made of 0.55M dimethyl sulfoxide (DMSO), 0.7M ethylene glycol (EG), and 0.65M trehalose or 0.35M ectoine, 0.7M EG, and 0.65M trehalose in FHM20 medium for 5 min on ice. The FHM20 medium is FHM medium (Millipore) supplemented with 20% fetal bovine serum (FBS, RMBIO) and buffered by 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.2. Next, the mouse oocytes were transferred into a droplet of 30 µl vitrification solution made of 1.1M DMSO, 1.4M EG, and 1.3 M trehalose (DMSO-based CPA) or 0.7M ectoine, 1.4M EG, and 1.3 M trehalose (ectoine-based bio-inspired CPA) in FHM20 medium for 1 min on ice. The mouse oocytes were loaded into a quartz microcapillary (QMC) (Charles Supper Company) for vitrification. The oocyte-laden QMC was then plunged into liquid nitrogen and held in there for at least 1 min. Afterward, the QMC was transferred from liquid nitrogen into a solution consisting of 0.2 M trehalose in 1 × phosphate-buffered saline (PBS) at 37 °C to warm up the sample, as it has

been described in the literatures(Choi, *et al.*, 2015a, Choi, *et al.*, 2015b). After warming, the mouse oocytes were pushed out of the QMC and washed three times by transferring them consecutively in three droplets of 30 μ l of FHM20 at room temperature and then incubate them for 5 min.

Human oocyte vitrification—For human oocytes vitrification, they were used in Gardner's (G1; Vitrolife) medium20 that consists of 20% knockout serum replacement (KSR, Gibco) and buffered by HEPES at pH 7.2. We followed the same experimental procedures for human oocyte vitrification like mouse oocyte vitrification steps that used DMSO-based CPA and ectoine-based bio-inspired CPA. To check the viability of mouse and human oocytes after the vitrification procedure, the recovered oocytes were cultured at 37 °C for 10 min following the manufacture instructions and literatures (Choi, *et al.*, 2015a, Choi, *et al.*, 2015b) in FHM20 medium and G120 with 2 μ M calcein AM and 4 μ M ethidium homodimer (Invitrogen)/propidium iodide (PI; 10 μ g/ml) for live (green) and dead (read) staining, respectively.

Parthenogenetic activation of mouse oocytes

Vitrified mouse MII oocytes were parthenogenetically activated by culturing in KSOM supplemented with 10 mM SrCl₂ for 4 h at 37°C in 5% CO₂ in air and then washed three-times with FHM medium. They were cultured in KSOM at 37°C, 5% CO₂ in air and monitored under a phase contrast light microscope (Zeiss) for the formation of two-cells, four-cells, morula, and blastocysts at various times for up to 5 days.

Statistical analysis

A generalized linear model (PROC-GLM) and one-way ANOVA in a Statistical Analysis System (SAS) program was used for statistical analysis to determine the p-value between various treatments. The differences were taken as significant when the p-value was less than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Bio-inspired vitrification of oocytes

a) Essentials of experimental setup for minimum volume vitrification. Oocytes were loaded with ectoine-based CPA solution, and placed into quartz microcapillary (QMC) for vitrification. A photographic image showing the QMC used in this study and a zoom-in view showing four oocytes in the loaded zone of the QMC. **b)** QMC was submerged into liquid nitrogen (LN_2) for 1 min, and then rewarmed in phosphate-buffered saline (PBS) with 0.2M trehalose for 1 mins. **c)** The recovered oocytes were then evaluated for viability using Live/Dead staining after thawing. Scale bars represent: 50 μm (mouse oocyte); 100 μm (human oocyte). **d)** The functionality of viable mouse oocytes was demonstrated by embryonic development (from two cells to blastocyst) via parthenogenetic activation. It is worth mentioning that the embryonic development of human oocytes cannot be performed for research due to ethical and legal issues.

Figure 2. Viability assessment of recovered mouse oocytes after ectoine-based vitrification and rewarming

Phase (**a,c,e,g**) and fluorescence (**b,d,f,h**) micrographs for mouse metaphase II (MII) oocytes after vitrification using ectoine-based and DMSO-based (control) methods. The fluorescent micrographs showing live (**b,f**) and dead (**d,h**) oocytes. Scale bars=50µm. **i**) Table showing the viability (number and percentage (%)) of MII mouse oocytes after ectoine-based vitrification and rewarming.

Figure 3. Embryonic development of mouse oocytes via parthenogenetic activation after ectoine-based vitrification and rewarming

a) Embryonic development of recovered oocytes after ectoine-based vitrification compared to DMSO-based (positive control) and non-vitrified (negative control) groups. Scale bars=50 μ m. **b)** Table showing the embryonic development (number and percentage (%)) of mouse oocytes via parthenogenetic activation after ectoine-based vitrification and rewarming.

Figure 4. Viability assessment of recovered human oocytes after ectoine-based vitrification and rewarming

Phase (a,c,e,g) and fluorescence (b,d,f,h) micrographs for mouse metaphase II (MII) oocytes after vitrification using ectoine-based and DMSO-based (control) methods. The fluorescent micrographs showing live (b,f) and dead (d,h) oocytes. Scale bars=100μm. i) Table showing the viability (number and percentage (%)) of human oocytes after ectoine-based vitrification and rewarming.