Published in final edited form as:

Cryobiology. 2015 June ; 70(3): 269–272. doi:10.1016/j.cryobiol.2015.04.003.

Improved Low-CPA Vitrification of Mouse Oocytes Using Quartz Microcapillary

Jung Kyu Choi^{1,3}, Haishui Huang^{1,2}, and Xiaoming He^{1,3,4,*}

¹Department of Biomedical Engineering, The Ohio State University, Columbus, OH 43210

²Department of Mechanical and Aerospace Engineering, The Ohio State University, Columbus, OH 43210

³Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH 43210

⁴James Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210

Abstract

Cryopreservation by low-cryoprotectant (CPA) vitrification has the potential to combine all the advantages of the conventional high-CPA vitrification and slow-freezing approaches while avoiding their drawbacks. However, current low-CPA vitrification protocol for cryopreservation of oocytes requires a lengthy and multi-step procedure for unloading CPAs. In this study, we report a much-simplified procedure of using quartz microcapillary (QMC) for low-CPA vitrification of mouse oocytes with only one step for unloading CPAs. The immediate viability of oocytes after the improved low-CPA vitrification was determined to be more than 90%. Moreover, no significant difference was observed in terms of embryonic development from the two-cell to blastocyst stages between the fresh and vitrified oocytes after *in vitro* fertilization (IVF). This improved low-CPA vitrification technology has the potential for efficient cryopreservation of oocytes to preserve the fertility of mammals including humans for assisted reproductive medicine, maintenance of animal resource and endangered species, and livestock management.

Keywords

oocyte; cryopreservation; quartz microcapillary; low-CPA; vitrification; embryonic development

1. Introduction

Oocyte cryopreservation is important to the advancement of assisted reproductive medicine, maintenance of animal resources and endangered species, and livestock management. For example, cryopreservation of human oocytes can resolve the legal, moral, ethical, and

© 2015 Published by Elsevier Inc.

*Correspondence should be addressed to: Xiaoming He, Ph.D., Department of Biomedical Engineering, The Ohio State University, 1080 Carmack Road, Columbus, OH 43210, Phone: (614) 247-8759, Fax: (614) 292-7301, He.429@osu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

religious issues associated with human embryo cryopreservation, a routine procedure in clinics offering assisted reproduction today [3,6,21,33,38]. Moreover, oocyte cryopreservation can preserve future fertility of young and/or unmarried women who may lose gonadal function because of exposure to environmental/occupational hazards or aggressive medical treatments such as extirpative surgery, radiation therapy, and chemotherapy [14,25,31]. However, it is still not a commonly used clinical procedure [26-28]. With the availability of transgenic and knock-out techniques, it is estimated that 5000 new strains of mice will be created annually in the next decade [23]. Cryopreservation of germplasm of such a vast resource is needed to conserve the genome of these animals to avoid genetic drift, and more importantly to reduce the cost of maintenance and distribution. While the germplasm of some species can be cryopreserved with great efficiency, it is still difficult (particularly for oocytes) for some non-traditional animal models [7,8,15,17,23]. For livestock management in agriculture, although a large number of morphologically viable oocytes could be obtained after cryopreservation, the number of healthy offspring resulting from cryopreserved oocytes is much lower than that from cryopreserved embryos [2,18,19,24].

There are two conventional methods for oocyte cryopreservation: slow freezing and vitrification. Although a low concentration of penetrating (i.e., cell membrane permeable) cryoprotectant (CPA) such as DMSO (usually less than ~2 M, low-CPA) is required for slow freezing, injury to oocytes is inevitable as a result of the extensive cell dehydration during slowly freezing the extracellular water (i.e., freeze concentration) to reduce intracellular ice formation (IIF) [4,11,22]. For example, slow freezing has been shown to cause hardening of the zona pellucida [20] and damage to chromosome [1] of oocytes. The goal of vitrification is to minimize IIF and dehydration (or freeze concentration) altogether, but the conventional procedure of vitrification requires a high concentration of CPAs (up to ~8 M, high-CPA) that may induce significant osmotic and metabolic damage to cells including oocytes even in a short exposure of a few minutes [11,36,40]. For example, a high concentration of CPA has been shown to induce cytoskeletal alternations [5,35], dispersal of chromosome [30], and disassembly of spindles [32] in oocytes. Consequently, it is necessary to use multiple steps to gradually load and unload the high CPA to minimize osmotic and metabolic damage to cells during high-CPA vitrification, making it a complicated, lengthy, and stressful process [12,13,29].

More recently, low-CPA vitrification has been explored for cell cryopreservation by using various methods or devices [8-10,12,16,34,37,39], to achieve ultra-rapid cooling rate that minimizes IIF by reducing the time available for ice nucleation and growth. For example, the miniaturized quartz microcapillary (QMC) has been successfully used to achieve low-CPA vitrification of mouse mesenchymal stem cells (MSCs), mouse embryonic stem cells (ESCs), and mouse oocytes [12,16,39]. However, a lengthy and multi-step procedure was still used for unloading the CPAs when using QMC for low-CPA vitrification of mouse oocytes [16]. In this study, we further improve the QMC-based low-CPA vitrification by greatly simplifying the CPA unloading procedure to facilitate its wide application for cryopreservation of oocytes.

2. Materials and Methods

2.1. Animals and materials

Both male and 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 The Ohio State University and every effort was made to minimize animal suffering. All Materials were purchased from Sigma unless specifically mentioned otherwise.

2.2. Retrieval of 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 hours apart. Sixteen 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 M2 medium. To obtain clean oocytes, the COCs were incubated in M2 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 M2 medium. Metaphase II (MII) oocytes with a first polar body were transferred and cultured in KSOM medium (Millipore) at 37 °C and 5% CO₂ till before further experimental use.

2.3. *In vitro* fertilization (IVF) and embryo culture

To obtain sperm for IVF of oocytes, 6-8 week old male B6CBAF1 mice were euthanized by cervical dislocation and epididymides were collected by dissection. The epididymides were then placed in the central well of an IVF dish with KSOM medium. After making 5-7 longitudinal cuts using a syringe needle on each epididymis, the epididymides were incubated for 20 min at 37 °C in 5% CO₂ air to allow for sperm dispersion. The sperm suspensions were incubated for 1 h at 37 °C in 5% CO₂ air for capacitation. For IVF, 5 MII oocytes from the vitrification and fresh (control) groups were inseminated with 2×10^4 sperm in a droplet of 100 µl KSOM medium for 4.5 h. Fertilized oocytes were subsequently cultured in a drop of 50 µl of CZB medium at 37 °C in 5% CO₂ air. Development of the fertilized oocytes was monitored under a phase contrast light microscope (Nikon 80i) for the formation of 2-cell, 4-cell, morula, and blastocyst stage embryos at various intervals for up to 5 days.

2.4. Statistical analysis

A generalized linear model (PROC-GLM) 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.

3. Results and Discussion

Similar to the previous studies [12,16], we loaded CPA into mouse oocytes (at the metaphase II or MII stage) in two steps. This was done by first incubating the oocytes with a pre-equilibrium solution made of 4% ethylene glycol (EG) and 4% dimethyl sulfoxide

(DMSO) in FHM20 medium for 5 min at 4 °C. The FHM20 medium is FHM medium (Millipore) supplemented with 20% fetal bovine serum (FBS, Hyclone) and buffered by HEPES at pH 7.2. Next, the oocytes were transferred into a droplet of 30 µl vitrification solution made of 8% EG, 8% DMSO, and 1 M trehalose in FHM20 medium for 1 min at 4 °C. The total concentration of penetrating (i.e., cell membrane permeable) CPAs (i.e., EG and DMSO) in the vitrification solution was 2.5 M (1.4 M EG and 1.1 M DMSO). Afterward, the MII oocytes (4-5 in a group) were loaded into QMC one by one by using a mouth-controlled pipet. A typical image showing 4 oocytes loaded in the QMC is given in Figure 1. The QMCs (Charles Supper Company) used in this study are 200 µm in outer diameter with a wall thickness of 10 µm. After loading with oocytes, the QMCs were immediately plunged into liquid nitrogen and held there for 3 min that is long enough to cool the sample down to the temperature of liquid nitrogen [12]. For warming, the QMCs were immediately transferred from liquid nitrogen into a warming solution made of 0.2 M trehalose in isotonic phosphate-buffered saline (PBS) at 37 °C. The MII oocytes were then expelled into a droplet of 30 µl FHM20 medium for 3 min and further washed three times by consecutively transferring the oocytes into and out of three droplets of 30 µl FHM20 medium without stop. Therefore, we unloaded CPAs from the vitrified oocytes in one step rather than the tedious multi-step procedure of incubating in FHM20 medium with 0.5, 0.25, 0.125, and 0 M trehalose for 5 minutes in each of the solutions [16].

To check the viability of oocytes after the vitrification procedure, they were cultured at 37 °C for 10 min in M2 medium with 5 µM calcein AM and 5 µM ethidium homodimer (Invitrogen) to stain live and dead MII oocytes, respectively. Typical phase and fluorescence micrographs showing the morphology of live and dead oocytes after vitrification are given in Figure 2. As shown in Table 1, the viability of oocytes after the aforementioned vitrification procedure is high (33 out of 35 or 94%). We further performed *in vitro* fertilization of the vitrified MII oocytes and examined embryonic development of the fertilized MII oocytes. As shown in Table 2, the percentage of vitrified MII oocytes that developed to 2-cell, 4-cell, morula, and blastocyst stages is not significantly from that of control (i.e., fresh or non-vitrified) MII oocytes. No significant difference was observed either for the morphology of the 2-cell, 4-cell, morula, and blastocyst stage embryo between the vitrified and control groups (Figure 3).

The simple procedure for low-CPA vitrification of the large mouse oocytes in this study is almost the same as that previously reported for small mouse ESCs [12], except that different CPAs were used and the warming solution was at room temperature in the previous study instead of 37 °C in this work. Although warming the vitrified sample at 37 °C might not be as convenient as room temperature, it can also be easily conducted by utilizing the instruments (e.g., incubators and water bath) in biomedical laboratories and clinical facilities to maintain 37 °C for mammalian cell and tissue culture and the normal body temperature of humans.

In summary, we developed a simple and convenient procedure with only one step for unloading the CPAs for low-CPA vitrification of mouse oocytes using QMCs. We checked the oocyte viability and embryonic development by *in vitro* fertilization (IVF) following vitrification. Our results show that the QMC-based low-CPA vitrification with the much-

simplified procedure is a viable technology for effective cryopreservation of mammalian oocytes, which may be useful for preserving the fertility of mammals including humans for assisted reproductive medicine, maintenance of animal resource and endangered species, and livestock management.

Acknowledgments

This work was partially supported by a grant from NIH (R01EB012108). The authors would like to thank Jenna Dumbleton for proofreading this manuscript.

References

- [1]. Bernard A, Fuller BJ. Cryopreservation of human oocytes: a review of current problems and perspectives. *Hum Reprod Update*. 1996; 2:193–207. [PubMed: 9079413]
- [2]. Day BN. Reproductive biotechnologies: current status in porcine reproduction. *Anim Reprod Sci*. 2000; 60-61:161–172. [PubMed: 10844192]
- [3]. Deepinder F, Agarwal A. Technical and ethical challenges of fertility preservation in young cancer patients. *Reprod Biomed Online*. 2008; 16:784–791. [PubMed: 18549687]
- [4]. Diller KR. Bioheat and mass transfer as viewed through a microscope. *J Biomech Eng*. 2005; 127:67–84. [PubMed: 15868790]
- [5]. Egerszegi I, Somfai T, Nakai M, Tanihara F, Noguchi J, Kaneko H, Nagai T, Ratky J, Kikuchi K. Comparison of cytoskeletal integrity, fertilization and developmental competence of oocytes vitrified before or after in vitro maturation in a porcine model. *Cryobiology*. 2013; 67:287–292. [PubMed: 23993921]
- [6]. Eroglu A, Toner M, Toth TL. Beneficial effect of microinjected trehalose on the cryosurvival of human oocytes. *Fertil Steril*. 2002; 77:152–158. [PubMed: 11779606]
- [7]. Fray MD. Biological methods for archiving and maintaining mutant laboratory mice. Part I: conserving mutant strains. *Methods Mol Biol*. 2009; 561:301–319. [PubMed: 19504080]
- [8]. Gardner DK, Sheehan CB, Rienzi L, Katz-Jaffe M, Larman MG. Analysis of oocyte physiology to improve cryopreservation procedures. *Theriogenology*. 2007; 67:64–72. [PubMed: 17049589]
- [9]. Han X, Ma H, Jiao A, Critser JK. Investigations on the heat transport capability of a cryogenic oscillating heat pipe and its application in achieving ultra-fast cooling rates for cell vitrification cryopreservation. *Cryobiology*. 2008; 56:195–203. [PubMed: 18430413]
- [10]. He, X. Preservation of embryonic stem cells. In: Atwood, C., editor. *Methodological Advances in the Culture, Manipulation and Utilization of Embryonic Stem Cells for Basic and Practical Applications*. InTech Publisher; Vienna, Austria: 2011. p. 113-138.
- [11]. He X. Thermostability of biological systems: fundamentals, challenges, and quantification. *Open Biomed Eng J*. 2011; 5:47–73. [PubMed: 21769301]
- [12]. He X, Park EY, Fowler A, Yarmush ML, Toner M. Vitrification by ultra-fast cooling at a low concentration of cryoprotectants in a quartz micro-capillary: a study using murine embryonic stem cells. *Cryobiology*. 2008; 56:223–232. [PubMed: 18462712]
- [13]. Heng BC, Kuleshova LL, Basted SM, Liu H, Cao T. The cryopreservation of human embryonic stem cells. *Biotechnol Appl Biochem*. 2005; 41:97–104. [PubMed: 15588230]
- [14]. Jeruss JS, Woodruff TK. Preservation of fertility in patients with cancer. *N Engl J Med*. 2009; 360:902–911. [PubMed: 19246362]
- [15]. Kouba AJ, Vance CK. Applied reproductive technologies and genetic resource banking for amphibian conservation. *Reprod Fertil Dev*. 2009; 21:719–737. [PubMed: 19567216]
- [16]. Lee HJ, Elmoazzen H, Wright D, Biggers J, Rueda BR, Heo YS, Toner M, Toth TL. Ultra-rapid vitrification of mouse oocytes in low cryoprotectant concentrations. *Reprod Biomed Online*. 2010; 20:201–208. [PubMed: 20113958]
- [17]. Leibo SP, Songsasen N. Cryopreservation of gametes and embryos of non-domestic species. *Theriogenology*. 2002; 57:303–326. [PubMed: 11775977]

- [18]. Lewis IM, Peura TT, Trounson AO. Large-scale applications of cloning technologies for agriculture: an industry perspective. *Reprod Fertil Dev*. 1998; 10:677–681. [PubMed: 10612476]
- [19]. Lonergan P. State-of-the-art embryo technologies in cattle. *Soc Reprod Fertil Suppl*. 2007; 64:315–325. [PubMed: 17491156]
- [20]. Lucena E, Bernal DP, Lucena C, Rojas A, Moran A, Lucena A. Successful ongoing pregnancies after vitrification of oocytes. *Fertil Steril*. 2006; 85:108–111. [PubMed: 16412739]
- [21]. Mandelbaum J, Belaisch-Allart J, Junca AM, Antoine JM, Plachot M, Alvarez S, Alnot MO, Salat-Baroux J. Cryopreservation in human assisted reproduction is now routine for embryos but remains a research procedure for oocytes. *Hum Reprod*. 1998; 13(Suppl 3):161–174. discussion175–167. [PubMed: 9755422]
- [22]. Mazur P. Freezing of living cells: mechanisms and implications. *Am J Physiol*. 1984; 247:C125–142. [PubMed: 6383068]
- [23]. Mazur P, Leibo SP, Seidel GE Jr. Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. *Biol Reprod*. 2008; 78:2–12. [PubMed: 17901073]
- [24]. Nagashima H, Fujimura T, Takahagi Y, Kurome M, Wako N, Ochiai T, Esaki R, Kano K, Saito S, Okabe M, Murakami H. Development of efficient strategies for the production of genetically modified pigs. *Theriogenology*. 2003; 59:95–106. [PubMed: 12499021]
- [25]. Nieman CL, Kinahan KE, Yount SE, Rosenbloom SK, Yost KJ, Hahn EA, Volpe T, Dilley KJ, Zoloth L, Woodruff TK. Fertility preservation and adolescent cancer patients: lessons from adult survivors of childhood cancer and their parents. *Cancer Treat Res*. 2007; 138:201–217. [PubMed: 18080667]
- [26]. Porcu E, Venturoli S. Progress with oocyte cryopreservation. *Curr Opin Obstet Gynecol*. 2006; 18:273–279. [PubMed: 16735826]
- [27]. M. Practice Committees of American Society for Reproductive; T. Society for Assisted Reproductive. Mature oocyte cryopreservation: a guideline. *Fertil Steril*. 2013; 99:37–43. [PubMed: 23083924]
- [28]. Quaas AM, Melamed A, Chung K, Bendikson KA, Paulson RJ. Egg banking in the United States: current status of commercially available cryopreserved oocytes. *Fertil Steril*. 2013; 99:827–831. [PubMed: 23200684]
- [29]. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at –196 degrees C by vitrification. *Nature*. 1985; 313:573–575. [PubMed: 3969158]
- [30]. Saunders KM, Parks JE. Effects of cryopreservation procedures on the cytology and fertilization rate of in vitro-matured bovine oocytes. *Biol Reprod*. 1999; 61:178–187. [PubMed: 10377047]
- [31]. Stillerman KP, Mattison DR, Giudice LC, Woodruff TJ. Environmental exposures and adverse pregnancy outcomes: a review of the science. *Reprod Sci*. 2008; 15:631–650. [PubMed: 18836129]
- [32]. Tamura AN, Huang TT, Marikawa Y. Impact of vitrification on the meiotic spindle and components of the microtubule-organizing center in mouse mature oocytes. *Biol Reprod*. 2013; 89:112. [PubMed: 24025740]
- [33]. Tucker M, Morton P, Liebermann J. Human oocyte cryopreservation: a valid alternative to embryo cryopreservation? *Eur J Obstet Gynecol Reprod Biol*. 2004; 113(Suppl 1):S24–27. [PubMed: 15041126]
- [34]. Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reprod Biomed Online*. 2006; 12:779–796. [PubMed: 16792858]
- [35]. Vincent C, Johnson MH. Cooling, cryoprotectants, and the cytoskeleton of the mammalian oocyte. *Oxford reviews of reproductive biology*. 1992; 14:73–100. [PubMed: 1437216]
- [36]. Yang G, Zhang A, Xu LX, He X. Modeling the cell-type dependence of diffusion-limited intracellular ice nucleation and growth during both vitrification and slow freezing. *Journal of Applied Physics*. 2009; 105:114701.
- [37]. Yavin S, Arav A. Measurement of essential physical properties of vitrification solutions. *Theriogenology*. 2007; 67:81–89. [PubMed: 17070573]

- [38]. Zeilmaker GH, Alberda AT, van Gent I, Rijkman CM, Drogendijk AC. Two pregnancies following transfer of intact frozen-thawed embryos. *Fertil Steril*. 1984; 42:293–296. [PubMed: 6745463]
- [39]. Zhang W, Yang G, Zhang A, Xu LX, He X. Preferential vitrification of water in small alginate microcapsules significantly augments cell cryopreservation by vitrification. *Biomed Microdevices*. 2010; 12:89–96. [PubMed: 19787454]
- [40]. Zhao G, Takamatsu H, He XM. The effect of solution nonideality on modeling transmembrane water transport and diffusion-limited intracellular ice formation during cryopreservation. *Journal of Applied Physics*. 2014; 115:144701, 144713. [PubMed: 25316951]

Figure 1.
A typical picture of the quartz microcapillary (QMC) used in this study for vitrification together with a zoom-in view showing four oocytes loaded in the QMC. Scale bar: 10 mm.

Figure 2.

Typical phase and fluorescence micrographs showing the morphology of live and dead MII oocytes after vitrification: The live and dead oocytes are stained green and red, respectively. Scale bar: 40 μm .

Figure 3.

Typical micrographs showing 2-cell, 4-cell, morula, and blastocyst stage embryos during embryonic development of fresh and vitrified MII oocytes after *in vitro* fertilization (IVF): both the fresh and vitrified oocytes could develop to the blastocyst stage with no significant difference. Scale bar: 40 μ m.

Table 1

Viability of MII oocytes from B6CBAF1 mice after vitrification: The MII oocytes were isolated from two animals.

Number (%) of MII oocytes	Number (%)of	
	Live oocytes	Dead oocytes
35	33 (94)	2 (6)

Table 2

Embryonic development of *in vitro* fertilized oocytes of B6CBAF1 mice either without (control) or with vitrification: A total of six animals were used to isolate the MII oocytes.

Group	Number of MII oocytes	Number (%) of 2-cell embryos	Number (%) ^a of embryos developed to		
			4-cell	Morula	Blastocyst
Control	45	31 (68)	27 (87)	15 (55)	13 (41)
Vitrification	46	26 (56)	21 (80)	11 (42)	8 (30)

^aPercentage of total 2-cell