Cryobiology 68 (2014) 18–28

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Optimization of cryoprotectant loading into murine and human oocytes ☆

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ARTICLE INFO

Article history:
Received 14 August 2013
Accepted 7 November 2013
Available online 15 November 2013

Keywords:
Cryopreservation
Cryoprotectant
Dimethyl sulfoxide
DMSO
Propylene glycol
Simplex optimization
Oocyte
Human
Freezing
Vitrification

ABSTRACT

Loading of cryoprotectants into oocytes is an important step of the cryopreservation process, in which the cells are exposed to potentially damaging osmotic stresses and chemical toxicity. Thus, we investigated the use of physics-based mathematical optimization to guide design of cryoprotectant loading methods for mouse and human oocytes. We first examined loading of 1.5 M dimethyl sulfoxide (Me_2SO) into mouse oocytes at 23 °C. Conventional one-step loading resulted in rates of fertilization (34%) and embryonic development (60%) that were significantly lower than those of untreated controls (95% and 94%, respectively). In contrast, the mathematically optimized two-step method yielded much higher rates of fertilization (85%) and development (87%). To examine the causes for oocyte damage, we performed experiments to separate the effects of cell shrinkage and Me_2SO exposure time, revealing that neither shrinkage nor Me_2SO exposure single-handedly impairs the fertilization and development rates. Thus, damage during one-step Me_2SO addition appears to result from interactions between the effects of Me_2SO toxicity and osmotic stress. We also investigated Me_2SO loading into mouse oocytes at 30 °C. At this temperature, fertilization rates were again lower after one-step loading (8%) in comparison to mathematically optimized two-step loading (86%) and untreated controls (96%). Furthermore, our computer algorithm generated an effective strategy for reducing Me_2SO exposure time, using hypotonic diluents for cryoprotectant solutions. With this technique, 1.5 M Me_2SO was successfully loaded in only 2.5 min, with 92% fertilizability. Based on these promising results, we propose new methods to load cryoprotectants into human oocytes, designed using our mathematical optimization approach.

Introduction

Oocyte cryopreservation may help to preserve future fertility of women who face cancer/extirpative therapy or want to delay childbearing years. It would also avoid many legal and ethical issues associated with embryo freezing. Furthermore, cryobanking of oocytes may help conservation of endangered species and improvement of livestock breeding.

Although the first successful cryopreservation of mammalian and human oocytes was achieved in the 1970s [55,76] and mid-1980s [13], respectively, oocyte cryopreservation has proven to be challenging due to the diversity of mechanisms leading to cryoinjury. Known manifestations of oocyte cryoinjury include intracellular ice formation [45], cell lysis [6], osmotic stress [1], disruption of cytoskeleton and spindle microtubules [18,75], premature exocytosis of cortical granules and zona hardening [12,64], parthenogenetic activation [65,68,73], and polyploidy [2,18,25]. Only after a decade of additional research and implementation of intracytoplasmic sperm injection (ICSI), it has become possible to mitigate some of these cryoinjury mechanisms, and to reproduce the initial success of human oocyte cryopreservation [40,60,71]. Subsequently, increasingly encouraging results have been reported with both ice-assisted slow-cooling techniques [9–11,27,39,56] and ice-free vitrification methods [15,41,66,77]. More recently, clinical success rates similar to those of unfrozen controls have been reported by three groups using an open-system vitrification approach requiring minimum sample volume and extremely fast cooling/warming rates [4,17,63]. However, this vitrification approach is particularly prone to handling issues and devitrification due to the minimal sample volume (less than 1 μl) and low concentrations (~30%) of intracellular cryoprotectant

☆ *Statement of funding:* This work was supported by a grant from the National Institute of Child Health and Human Development (Grant No. R01HD049537).

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0011-2240/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cryobiol.2013.11.002

additive (CPA), while direct contact with LN_2 in open systems poses a serious biosafety risk [8,28,46,70]. Moreover, the open-system vitrification approach is highly dependent on operator skill, and thawed oocytes must be fertilized by ICSI [4,17,63]. Although slow-cooling techniques are usually safer and not associated with a biosafety risk, their overall success rates in multicenter studies remain significantly lower than that of controls [11,56]. Therefore, further research is needed to improve the efficiency, reliability, and biosafety of the currently used cryopreservation techniques.

One of the critical steps in cryopreserving oocytes is the loading of permeating CPAs such as dimethyl sulfoxide (Me_2SO), propylene glycol (PROH), and ethylene glycol (EG), which may result in severe osmotic perturbations and CPA toxicity depending on the specifics of the experimental protocol. It is known that such insults compromise oocyte viability and developmental capacity [1,32,34,53,65,68]. Consequently, optimization of CPA loading procedures is of importance to maximize the probability of success in oocyte cryopreservation.

Although it has long been recognized that mathematical models of membrane transport can be used to guide the design of CPA loading and removal methods, the conventional approach to protocol development has been limited to prevention of osmotic stress (i.e., avoiding excessive cell volume excursions) without consideration of chemical toxicity due to prolonged CPA exposure [5,23,33,48,50,52]. Moreover, these early studies focused on the formulation of CPA solutions used for step-wise addition and elution, whereas the exposure time to each solution was not optimized [5,23,33,48,50,52]. In contrast, we recently introduced an optimization cost function to estimate the accumulation of toxicity damage, and used a Nelder–Mead simplex algorithm to automatically select the optimal times of exposure to each CPA solution as well as the optimal solution compositions [37]. Thus, we were able to design step-wise addition and removal procedures for rhesus monkey oocytes, resulting in protocols with total CPA exposure time comparable to or faster than that of one-step methods, but with significantly reduced osmotic stress [37]. However, we were unable experimentally evaluate our computer-optimized procedures, because primate oocytes are scarce, and all rhesus monkey oocytes collected in our previous investigation were used for measurement of the biophysical properties required to simulate the cell response.

Thus, in the present study, our goal was to experimentally test the hypothesis that oocyte viability and function will be higher when CPA addition is performed using a two-step process optimized by simultaneously limiting osmotic stress and CPA toxicity, than when CPA addition is performed in a single step (exposing oocytes directly to the full-strength CPA solution). To accomplish this, we employed our optimization approach to develop minimally damaging CPA addition procedures for mouse metaphase II (M II) oocytes. The computer-generated CPA loading protocols were realized experimentally, allowing the predictions to be validated, and demonstrating that optimized CPA addition methods can yield fertilization and embryonic development rates similar to those of untreated controls. Moreover, additional experimental tests were performed to shed light on the mechanisms of oocyte damage resulting from non-optimized CPA loading. Given the success demonstrated with mouse oocytes, we also used the computer models to design optimal processes for loading of PROH into human oocytes.

Materials and methods

Reagents and media

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated. Bicarbonate-buffered Hypermedium [19,20] supplemented with 4 mg/ml bovine serum albumin (BSA) and 50 mg/ml gentamycin served as a culture medium. For manipulation of oocytes and embryos under air, Hypermedium was buffered with 15 mM HEPES. Our previous studies showed that the Hypermedium supports mouse embryonic development and fertilized mouse eggs cultured in Hypermedium can develop into healthy mice when transferred to pseudo pregnant females [19,20]. Before culturing oocytes and embryos, drops of the Hypermedium were overlaid by embryo-tested mineral oil and equilibrated overnight under a humidified atmosphere of 5% CO_2 in air at 37 °C.

Galactose solutions used in osmotic shock experiments were prepared by adding 0.1 M, 0.3 M, or 0.5 M galactose to isotonic Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) containing 4 mg/ml BSA.

To dilute Me_2SO to specified concentrations as required for CPA loading (0.738 M, 0.750 M, 0.976 M, 1.40 M, and 1.50 M), we used either the BSA-supplemented isotonic Ca^{2+}/Mg^{2+}-free PBS or one of two hypotonic buffers as the aqueous diluent. One hypotonic buffer formulation, designated "hypo-PBS", comprised Ca^{2+}/Mg^{2+}-free PBS that was diluted in ddH_2O as well as fetal bovine serum (FBS) to yield a calculated osmolarity of ~55 mOsmol/L (and a final FBS content of 10% v/v). Another hypotonic diluent, designated "hypo-NaCl", was prepared by adding NaCl and either FBS (10% v/v) or BSA (4 mg/ml) to a 15 mM Hepes buffer until the calculated osmolarity reached ~55 mOsmol/L. These hypotonic buffer formulations were designed so that the final salt osmolarity would be 50 mOsmol/L after addition of 1.4 M (i.e., 9.98% v/v) Me_2SO. To check this, the buffer osmolalities were measured by freezing-point depression osmometry after addition of an equivalent volume (9.98% v/v) ddH_2O, and confirmed to be 50 ± 5 mOsmol/kg (which is equal to 50 ± 5 mOsmol/L). For CPA removal, diluted Me_2SO solutions (0.5 M and 1.0 M), either with or without 0.25 M sucrose, were prepared using the isotonic Ca^{2+}/Mg^{2+}-free PBS with BSA only.

Oocyte isolation

All animal experiments were approved by the Institutional Animal Care and Use Committee at the Medical College of Georgia/Georgia Regents University. M II oocytes were obtained from 4–8 week-old B6D2F1 (C57BL/6NCr X DBA/2NCr; NCI, Frederick, MD) hybrid mice. Superovulation and collection of M II oocytes were carried out as described elsewhere [20]. To remove cumulus cells, the oocyte-cumulus masses were exposed to 120 IU/ml of bovine testis hyaluronidase (Type IV-S) at ambient temperature for 3–4 min. Next, the oocytes were washed in HEPES-buffered Hypermedium twice and then transferred to the Hypermedium for recovery before experimentation. For each experiment, M II oocytes were typically isolated from three or more female mice, pooled, and then randomly distributed among the experimental groups.

Simulation of oocyte response to cryoprotectant solutions

Oocyte volume and intracellular cryoprotectant concentration changes in response to molecular transport across the oolemma were simulated using custom software developed by one of us (JOMK) in the MATLAB® programming language (The MathWorks, Inc., Natick, MA). As previously described [37], the coupled transport of water and cryoprotectant additives (Me_2SO or PROH) across the cell membrane during cryoprotectant loading was described by a Jacobs-type two-parameter model [38], in which water chemical potential (i.e., the osmotic driving force) was estimated using an ideal-solution approximation.

For simulation of the response of mouse oocytes exposed to Me_2SO at 23 °C or 30 °C, published biophysical properties were