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Vitrification by Ultra-fast Cooling at a Low Concentration of Cryoprotectants in a Quartz Microcapillary: A Study Using Murine Embryonic Stem Cells

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Abstract

Conventional cryopreservation protocols for slow-freezing or vitrification involve cell injury due to ice formation/cell dehydration or toxicity of high cryoprotectant (CPA) concentrations, respectively. In this study, we developed a novel cryopreservation technique to achieve ultra-fast cooling rates using a quartz microcapillary (QMC). The QMC enabled vitrification of murine embryonic stem (ES) cells using an intracellular cryoprotectant concentration in the range used for slowing freezing (1–2 M). The cryoprotectants used included 2 M 1,2-propanediol (PROH, cell membrane permeable) and 0.5 M extracellular trehalose (cell membrane impermeable). More than 70% of the murine ES cells post-vitrification attached with respect to non-frozen control cells, and the proliferation rates of the two groups were similar. Preservation of undifferentiated properties of the pluripotent murine ES cells post vitrification cryopreservation was verified using three different types of assays: the expression of transcription factor Oct-4, the presentation of the membrane surface glycoprotein SSEA-1, and the elevated expression of the intracellular enzyme alkaline phosphatase. These results indicate that vitrification at a low concentration (2 M) of intracellular cryoprotectants is a viable and effective approach for the cryopreservation of murine embryonic stem cells.

Keywords

Cryopreservation; vitrification; 1,2-propanediol; trehalose; murine ES cells; quartz microcapillary; plastic straw

INTRODUCTION

Effective long-term storage (e.g., cryopreservation) of important mammalian cells such as oocytes, sperm, stem cells and their derivatives is critical to the success of cell based medicine

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such as tissue engineering, regenerative medicine, and assisted reproduction [16,28]. There are currently two approaches to achieve cryopreservation of mammalian cells: conventional slow freezing (i.e., with ice formation) and vitrification (i.e., without ice formation) [12,15,26,31, 36]. Although conventional slow freezing requires a low relatively nontoxic concentration of cryoprotectants (1–2 M), it is always associated with cell injury due to ice formation, concentration of solutes during freezing, and prolonged exposure to cryoprotectant and chilling temperatures between 10 and –40 °C [15,26,31]. Therefore, cryopreservation by slow freezing typically provides utilitarian but sub-optimal outcomes that are often incompatible with the development of innovative therapeutic strategies for modern medicine [15,20,22,26].

Cryopreservation by vitrification avoids ice formation all together and has proven to be a promising alternative to conventional slow freezing techniques. Existing protocols for vitrification, however, require a very high concentration of cryoprotectants (CPAs, generally more than 4M) that is usually toxic to most mammalian cells such as stem cells, oocytes and sperm [11,13–15,20–22,35,36]. As a result, it is necessary to use multiple steps of cryoprotectant loading and dilution and maintain short exposure times with the high concentration cryoprotectant in each step to minimize injury, which could make the procedure complicated, stressful, and difficult to control in some situations such as the vitrification of oocytes [20,22]. Therefore, it is of great interest to develop a novel approach to achieve vitrification of mammalian cells using a low nontoxic concentration of cryoprotectants, which combines the advantages of the existing slow freezing and vitrification approaches while avoiding their shortcomings. Theoretically, this can be done by ultra-fast cooling ($>100,000$ °C/min) mammalian cells to a vitrified/glassy state at cryogenic temperatures. This is because the higher the cooling rate is, the lower the required CPA concentration for vitrification [2,4, 5,25,40] and even pure water can be vitrified when the cooling rate is higher than 10^6 °C/s [3,5].

Various cooling devices have been used for achieving high cooling rates (>1000 °C/min) for vitrification of important mammalian cells such as oocytes. These devices include the traditional French type plastic straw, the open pulled straw, the electron microscopy copper grid, and cryoloop as reviewed in references [15,22]. The cooling rates that can be achieved using these devices are usually lower than $50,000$ °C/min and therefore high and toxic concentrations of cryoprotectants are required. Considering that these available cooling devices have large working dimensions (>0.8 mm in diameter for straws) and/or are made of nonconductive materials such as plastics, we are interested in the enhancement of heat transfer to achieve higher cooling rates ($>100,000$ °C/min) for vitrification at a lower cryoprotectant concentration through the combined use of a thin-walled microcapillary (<0.4 mm) with highly conductive wall materials such as quartz.

In this study, we report the development of an effective cryopreservation technique using a quartz microcapillary (QMC, outer diameter = 0.2 mm, wall thickness = 0.01 mm) to achieve ultra-fast ($>100,000$ °C/min) vitrification at a low and nontoxic level of cryoprotectants (2 M 1,2-propanediol and 0.5 M extracellular trehalose). We investigated this technique first by thermal modeling and solution studies. We then tested the efficacy of QMC-assisted vitrification for maintaining murine embryonic stem cell viability, attachment, proliferation, and pluripotency. The results from this study indicate that QMC circumvents the physical limitations which previously hindered vitrification at low cryoprotectant concentrations. Our results also show that QMC-assisted vitrification is an effective technique for cryopreserving murine embryonic stem cells and potentially other types of sensitive and important mammalian cells.

MATREIAL AND METHODS

1. Murine embryonic stem (ES) cell culture

For the purpose of evaluating the maintenance of embryonic stem cell pluripotency, a green fluorescent protein (GFP)-reporter cell system was used. The R1 murine ES cell line which expresses GFP under control of the Oct4 promoter was kindly provided by Andras Nagy (U. Toronto) [34]. The ES cell maintenance media consisted of Knockout DMEM supplemented with 15% Knockout Serum Replacement (Invitrogen, Carlsbad, CA) containing 1000U/ml LIF (Chemicon, Temecula, CA). Feeder layer-free ES cells were continually passaged in 0.1% gelatin-coated 75 cm² T-flasks in 5% CO₂ humidified air at 37°C.

2. Vitrification devices

The devices used in this study include capillary tubes with various dimensions and wall materials. A micro-drop on a copper electron-microscope grid was also considered in the thermal analysis. The configuration of a semi-spherical micro-drop (0.5μl) on a copper grid is referred to as 'grid' in this study. The capillary tubes used include the traditional French type straw (TS, Wipak Medical, Germany), the open pulled straw (OPS, Wipak Medical, Germany) and the thin walled quartz micro-capillary (QMC, Wolfgang Muller Glas Technik, Germany) as shown in Figure 1. Also shown in this figure is the so called superfine open pulled straw (SOPS, Wipak Medical, Germany) which is not significantly different from the open pulled straw either visually or in terms of vitrification capability [8]. A summary of the dimensions of the above-mentioned devices is given in Table 1. The thin walled quartz micro-capillary is transparent and has an inner diameter comparable to that of a human oocyte (~150μm on average), which is much smaller than that of the other devices shown in Figure 1.

3. Thermal analysis

In order to determine the properties of the quartz micro-capillary system that are most important for achieving ultra-fast cooling, we developed a thermal model. The intent of the model is to provide a rough estimate for the cooling rate and to explore the effects of different system parameters on that rate. A schematic of the heat transfer problem considered in this study is given in Figure 2. Due to symmetry of the geometry (i.e., the cylindrical capillary tube and the hemi-spherical micro-drop) and boundary conditions, a 2D axisymmetric thermal model described by Eq. 1 was used to predict the transient thermal distribution in the system:

$$\rho_i c_i \frac{\partial T}{\partial t} = \frac{\partial}{\partial z} \left(k_i \frac{\partial T}{\partial z} \right) + \frac{1}{r} \frac{\partial}{\partial r} \left(r k_i \frac{\partial T}{\partial r} \right) \quad (1)$$

where T is temperature, r is radial coordinate, z is axial coordinate, ρ is density, c is specific heat, k is thermal conductivity and the subscript 'i' represents either the vitrification solution or capillary wall. No phase change from water to ice was considered in the model since the goal of this study is to vitrify the cryoprotectant (CPA) laden solutions. If the solutions are successfully vitrified, then no ice will be formed. Convective boundary conditions were applied on the external boundaries that have direct contact with the cryogenic fluid (i.e., liquid nitrogen here):

$$-k_i \frac{\partial T}{\partial n} = h(T - T_{LN2}) \quad (2)$$

where h is the average convective boiling heat transfer coefficient between the external boundaries and liquid nitrogen, n is the outward normal of the external boundary surface, and

the subscript LN₂ represents liquid nitrogen. The boundary conditions on all other external surfaces without direct contact with liquid nitrogen were taken to be adiabatic. The boiling heat transfer coefficient was taken to be 10,000 W m⁻² °C⁻¹ in the thermal analysis, unless specified otherwise.

A summary of the thermal properties used in the thermal model is given in Table 2. To our knowledge, temperature dependent thermal properties of subcooled and vitrified water are by and large unknown at this time. Therefore, constant thermal properties calculated according to an approach given in [18] based on the thermal property of water at room temperature and water mass fraction in the solution were used. The partial differential equation (PDE) given in Equation 1 together with the boundary conditions was solved numerically using a commercially available finite element-based PDE solver package (FEMLAB v3.0, COMSOL, Inc., Burlington, MA). The computational domain was discretized using the built-in Lagrange-quadratic element in the FEMLAB element library. The element size was refined until the predicted temperatures is judged to be not size dependent (i.e., the first decimal point of the predicted temperatures does not vary on all nodes). As a result, the element size was as small as 5 µm. The time step was set to as small as 10⁻⁴s. The absolute convergence of temperature was set to be 0.01°C.

With the transient thermal distribution determined from thermal modeling, the average transient thermal history in the whole cryopreservation solution domain was calculated in FEMLAB using the following equation:

$$T_a = \left(\int_0^{r_s} \int_0^{z_s} 2\pi r T dr dz \right) / V_s \quad (3)$$

where V represents volume and the subscripts a and s represent average and solution, respectively. The cooling rate reported in this study was calculated as the ratio of the temperature difference between 0 and -130°C to the time required to reach -130°C from 0°C, which was determined from the average transient thermal history in the solution predicted using Eq. 3. We are interested in the cooling rate between 0 and -130°C, because this temperature range covers the freezing/melting points and glass transition temperatures of aqueous solutions for cryopreservation. Therefore, vitrification should be assured if the cooling rate is high enough to avoid ice formation between 0 and -130°C.

4. Apparent Vitrification of CPA Laden Solutions

One convenient way for determining non-vitrification is the appearance of opacity (or visible ice formation) when cooling solutions below their freezing point. If there is no observable opacity, it is called apparent vitrification. Although opacity could be easily identified in the traditional plastic straw and the open pulled straw by naked eyes, it is very difficult to do so for the quartz microcapillary due to its small dimension. Therefore, an experimental setup shown in Figure 3 was designed for visualization of opacity in the quartz micro-capillary during cooling by directing two focused lights generated from two fiber optic lamps on the capillary held against a dark background. Shown in the figure are two quartz microcapillaries loaded with solutions of different concentrations of 1,2-propanediol (PROH, Sigma, St Louis, MO) after plunging into liquid nitrogen. The top one, loaded with 2 M 1,2-propanediol in FHM (a HEPES-buffered physiological salt solution from Specialty Media, Lavallette, NJ), is clear, while the bottom one, with 1M 1,2-propanediol in FHM, is opaque. The solution in the top straw was probably vitrified while the solution in the bottom straw definitely was not. This setup was used to determine the threshold (minimum) CPA concentration required to completely avoid opacity during cooling of various CPA laden solutions. Three different CPAs were used including ethylene glycol (EG, Sigma, St Louis, MO), 1,2-propanediol and their

combination at a 1:1 ratio by volume. Various total CPA concentrations from 12% to 30% by volume with 2% increments were studied. Since 0.2-1M sucrose or trehalose has been used in essentially all studies for cryopreservation of mammalian oocytes using either the slow freezing or vitrification approaches, further opacity studies were performed using the quartz micro-capillary for solutions with various concentrations of sucrose (Sigma, St Louis, MO)/trehalose (Ferro Pfanstiehl Laboratories, Inc., Waukegan, IL) and PROH in FHM and ES cell medium. The solutions were loaded into the straws or the quartz microcapillaries by either capillary action or using an empty syringe attached with a soft PEP polymer needle tip to pull from the larger end of the straws or micro-capillaries.

5. Cryopreservation of Murine Embryonic Stem Cells by vitrification

On the day of experiment, the attached murine embryonic stem cells were lightly trypsinized for 3–5 minutes and collected. The cells were then pelleted at 100×g for 5 minutes and resuspended in cold stem cell medium (on ice, ~ 4°C) for further use. For vitrification studies, the cells were spun down at 100×g for 3–5 minutes and resuspended in 1ml solution made of ES cell maintenance medium with 1.5M 1,2-propanediol for 10 minutes. The cells were then spun down at 100×g for 3–5 minutes, solution decanted, and resuspended in a solution made of ES cell maintenance medium with 2 M 1,2-propanediol and 0.5 M trehalose at 10×10^6 cells/ml for another 10 minutes before cooling. Cells were also cryopreserved using either 0.5 M trehalose or 2 M 1,2-propanediol alone for comparison. When using 0.5 M trehalose alone, the cells were suspended (10×10^6 cells/ml) and incubated in ES cell maintenance medium with 0.5 M trehalose for 10min and then loaded into the quartz micro-capillary for cooling. In the case of 2 M 1,2-propanediol only, cells were suspended and incubated in 1ml solution made of ES cell maintenance medium with 1.5 M 1,2-propanediol for 10 minutes, spun down, solution decanted, resuspended (10×10^6 cells /ml) and incubated in ES cell maintenance medium with 2 M 1,2-propanediol for another 10 minutes before cooling. The cell suspensions were kept at ~ 4°C throughout the loading procedure. The cell concentration in the last step of loading was always at 10×10^6 cells/ml. The solution volume for resuspending cells during all the other loading steps was always 1 ml. The cell concentration during those steps varied from day to day depending on the total amount of cells (~3–10 million) available on the day of experiments. The pH of all solutions was carefully adjusted to 7.2~7.4 and was maintained using HEPES buffer at a concentration of 15–20 mM. The cell suspension in the quartz micro-capillary was cooled by plunging the micro-capillary into liquid nitrogen using a technique that we found resulted in apparent vitrification of the solution at much lower CPA concentrations than straight plunging. Figure 2 illustrates the technique of plunging, in which the straw is held above the LN2 with its length parallel to the surface and then the tip of the straw is plunged by rotating the straw downward around the operator's wrist so that the tip is plunged at a speed of greater than 1 m/s ($V > \sim 1$ m/s, Figure 2). The speed of the tip was calculated based on the measured time of motion and distance traveled. This technique resulted in apparent vitrification at a much lower cryoprotectant concentration than that required for the technique of plunging the straw along its radial axis, indicated as z in the figure. The microcapillary was then left in liquid nitrogen for 3–5 minutes. The cryopreserved cell suspension was then warmed by plunging the quartz micro-capillary into 1x PBS with 0.2 M trehalose at room temperature. The cell suspension was then unloaded from the quartz micro-capillary by forcing warm ES cell maintenance medium with 0.2 M trehalose into the larger end (Figure 1) of the quartz micro-capillary with the aid of a syringe attached with a soft PEP polymer needle tip. The yield of this unloading procedure was found to be more than 90% when the cell suspension was collected in a ~10 μ l droplet by counting the cell numbers before and after the procedure. This high unloading efficiency probably is due to the hydrophilic nature of the quartz wall which has a low binding affinity with the lipophilic cell membrane. This low binding affinity prevents the cells from sticking on the inner surface of the capillary as might occur in plastic straws. The cells were then further processed for immediate viability,

attachment, and proliferation analysis. The non-frozen control cells were harvested from the same batch as those prepared for vitrification on the day of experiment. Therefore, they were of the same passage number as those cells for vitrification studies.

6. Cell Viability, Attachment and Proliferation Post Vitrification

The immediate cell viability post cryopreservation was assessed using the standard live/dead assay kit of fluorescent probes: calcein AM and ethidium homodimer (Invitrogen) to check the cell membrane integrity. To do this, the cell suspension in the quartz micro-capillary after warming up was expelled into a small droplet (~2–3 μ l) of warm ES cell maintenance medium with trehalose (0.2 M) and the fluorescent probes (9 μ M Calcein AM and 9 μ M ethidium homodimer) in a Petri dish and incubated for 5–10 minutes at 37°C for dye uptake. The cell containing droplet was then covered with a coverslip and the cell membrane integrity was evaluated using a Nikon Diaphot 300 epi-fluorescence inverted microscope (10x objective). Cells that excluded ethidium homodimer (red) and retained the fluorescent calcein (green) were counted as viable. The total number of cells under each field was determined using the corresponding phase field. At least fifteen randomly selected fields of view were used for each sample at each day. The fields were randomly selected starting on one edge of the coverslip by moving the microscope stage in a constant direction until the field of view hit the other end of the coverslip. Usually, at least fifteen fields in two perpendicular directions were randomly selected to eliminate as much as possible the effect of non-homogeneity of cell distribution on the method used to determine cell viability. The immediate cell viability was calculated as the ratio of the number of viable cells to the number of total cells per field (10x).

To check the cell viability at a longer time post vitrification cryopreservation, cell attachment was estimated at day 1 and cell and proliferation or growth was evaluated over a three day observation period. The cell suspension in the quartz micro-capillary after warming was expelled into 1 ml warm ES cell maintenance medium with 0.2 M trehalose and incubated for 10–15 minutes at 37°C. A total of six micro-capillaries with a 4 cm cell suspension column in the capillary were used for each experimental condition. The 1ml ES cell suspension was then transferred into 9 ml warm fresh stem cell maintenance medium and incubated for another 10–15 minutes. The cells were then spun down, resuspended in 1.5ml fresh ES cell maintenance medium at room temperature, and cultured in a 35mm Petri dish coated with 10 μ g/ml human fibronectin (Chemicon) for further study. Proliferation was assessed by counting the total number of cells per field of view (10x) for both the control and cryopreserved cells cultured on fibronectin-coated Petri dishes. At least fifteen randomly selected fields of view were used for each sample. The attachment efficiency was calculated as the ratio of the total number of cells per field of a cryopreserved sample to that of the control non-frozen sample at day 1. The effect of exposure to the cryoprotectant on membrane integrity, attachment efficiency, and proliferation was checked using cells went through all the loading/unloading procedures without cooling and warming against fresh cells. The immediate cell viability judged by membrane integrity was found to be essentially 100% for both cell groups. No visible difference was noticeable between the two groups of cells either in term of attachment and proliferation (data not shown). Therefore, fresh cells without cryopreservation were seeded at the same total cell concentration as control for the attachment and proliferation studies. Using fresh cells as control can account for the potential loss of dead and viable cells during centrifugation and avoid the underestimation of cell injury in cryopreservation.

7. Undifferentiated Properties of Pluripotent ES cells Post Vitrification

To determine whether the embryonic stem cells retained the undifferentiated properties as pluripotent cells post cryopreservation, we performed three different types of assays that are characteristic to the murine embryonic stem cells: the expression of transcription factor Oct-4, the expression of membrane surface glycoprotein SSEA-1, and the elevated expression of the

enzyme alkaline phosphatase. For immunofluorescence staining of SSEA-1, ES cells were fixed using 4% paraformaldehyde, permeabilized with 0.4% Triton X-100, and blocked against non-specific binding with 2% BSA and donkey serum. Monoclonal antibody against SSEA-1 (clone MC-480) was purchased from Chemicon. Antibody localization of SSEA-1 was performed using a Texas Red conjugated goat anti-mouse F(ab')₂ fragment antibody (Rockland, Gilbertsville, PA). Mounting medium containing DAPI (Vector Labs, Burlingame, CA) applied to the cells before observation. The histochemical staining of alkaline phosphatase was performed by incubating naphthol AS-BI phosphate and fast red violet solutions (Chemicon) with 4% paraformaldehyde-fixed ES cells for 15 minutes.

RESULTS

1. Thermal Analysis of Cooling Rates

It is notable that the boundary boiling heat transfer coefficient has a significant effect on cooling rate (Figure 4A), especially when it is lower than $100,000 \text{ W m}^{-2} \text{ }^{\circ}\text{C}^{-1}$ for the quartz micro-capillaries. Since all of the calculated cooling rates are much lower than that needed for the vitrification of pure water (roughly 1 Million $^{\circ}\text{C}/\text{sec}$), our model indicates that in order to minimize CPA it is very important to plunge the quartz micro-capillary as fast as possible into the cryogenic medium (liquid nitrogen) during cooling to create a forced impinging convective flow of liquid nitrogen around the quartz microcapillary to enhance the boiling heat transfer coefficient at the boundary (Figure 2). It was reported that the heat transfer coefficient for such a forced convective boiling flow of liquid nitrogen are generally higher than $2000 \text{ W m}^{-2} \text{ }^{\circ}\text{C}^{-1}$ and can be higher than $10,000 \text{ W m}^{-2} \text{ }^{\circ}\text{C}^{-1}$ [24,33,39,41]. When the boiling heat transfer coefficient is in the range of 2,000 to $10,000 \text{ W m}^{-2} \text{ }^{\circ}\text{C}^{-1}$, the predicted cooling rates for the open pulled straw and the traditional straw are around 20,000 and 3,000 $^{\circ}\text{C}/\text{min}$, respectively. These cooling rates are very similar to those for open pulled straw ($\sim 20,000 \text{ }^{\circ}\text{C}/\text{min}$) and traditional straw ($\sim 2500 \text{ }^{\circ}\text{C}/\text{min}$) measured by Vajta et al [42], which indicates the validity of the thermal model established in this study for estimating roughly the cooling rate and exploring the effects of different system parameters on that rate. The predicted cooling rate for the quartz microcapillary is more than $100,000 \text{ }^{\circ}\text{C}/\text{min}$ when the boiling heat transfer is more than $2000 \text{ }^{\circ}\text{C}/\text{min}$. Uncertainty in the heat transfer coefficient is one of the primary reasons a precise numerical model of heat transfer is not presented, but in order to explore the potential of the quartz microcapillary for augmenting heat transfer, we chose a heat transfer coefficient of $10,000 \text{ W m}^{-2} \text{ }^{\circ}\text{C}^{-1}$ for further thermal analysis. Given such a heat transfer coefficient, Figure 4A shows that the quartz micro-capillary results in a much higher cooling rate than the other available devices. The cooling rate of the quartz micro-capillaries is approximately one order of magnitude higher than both that of the grid design and the open pulled straw and two orders of magnitude higher than that of the traditional straw. This enhancement in heat transfer is due to the small inner diameter, thin wall, and highly conductive wall material of the quartz microcapillary in comparison to other devices (see Table 1 and Table 2 for comparison quantitatively). The effect of capillary dimensions including the inner diameter and wall thickness on cooling rate is further shown in Figure 4B and 4C for two different wall materials: quartz (B) and plastic (C), respectively. It is clear from the figures that the cooling rate increases in a very strong nonlinear fashion with the decrease of the inner diameter for both materials, especially when the inner diameter is less than 0.4 mm. The cooling rate also increases significantly with the decrease of wall thickness and this effect is more apparent when the inner diameter is less than 0.4 mm for both materials. These results indicate the importance of adopting micro-machined small and thin capillaries to achieve high cooling rates when plunging the microcapillaries into liquid nitrogen. Figure 4D further shows the effect of capillary wall material on cooling rate for a capillary with an inner diameter of $180 \text{ }\mu\text{m}$ and a wall thickness of $10 \text{ }\mu\text{m}$. It is clear that the cooling rate increases when using more conductive material by comparing plastics to quartz. However, the cooling rate reaches a plateau thereafter.

In other words, using a more conductive material than quartz does not measurably increase the cooling rate for a microcapillary with a wall thickness of 10 μ m and inner diameter no less than 180 μ m.

2. Apparent Vitrification of CPA Laden Solutions

In order to assess the effectiveness of the quartz microcapillaries in vitrification, further studies on vitrifying cryoprotectant laden solutions using the quartz microcapillary were performed. The threshold concentration for the complete absence of opacity of three different cryoprotectants (ethylene glycol, 1,2-propanediol, and their combination at a 1:1 ratio by volume) was determined for the traditional French type straw, the open pulled straw, the superfine open pulled straw, and the quartz micro-capillary. Typical experimental matrices are given in Table 3 for the open pulled straw and the quartz microcapillary. As shown in Figure 5, the threshold CPA concentration associated with the quartz microcapillary is at least 2 M less than that of the traditional straw and 1 M less than the open pulled straw (or the so-called superfine open pulled straw, which is not different from those of the open pulled straw, in agreement with the results reported in reference [8]). It is also shown that 1,2-propanediol (PROH) is superior to ethylene glycol (EG) in terms of the capability to achieve apparent vitrification. Most importantly, the threshold CPA concentration required for apparent vitrification is as low as 1.9M when using the combination of quartz microcapillary and 1,2-propanediol. Further experiments were also performed on FHM and ES cell medium with 0.2-1M trehalose plus 1.5M (~11% v), 2 M (~15% v) and 2.5 M (19% v) 1,2-propanediol. No opacity was observed after plunging the quartz microcapillary loaded with these solutions into liquid nitrogen. Based on these results, solutions of 2 M 1,2-propanediol (permeable CPA) and/or 0.5 M trehalose (impermeable CPA) were used in our vitrification studies of mammalian cells.

3. Immediate Cell Viability, Attachment Efficiency, Proliferation, and Undifferentiated Properties of ES Cells Post Vitrification

The immediate cell viability evaluated by cell membrane integrity post cryopreservation using various cryoprotectants is shown in Figure 6. Only a minimal number of cells (~20%) can survive using 2 M 1,2-propanediol alone. When adding 0.5 M trehalose into the vitrification solution, however, the cell viability increased to more than 80%. The immediate viability using 0.5 M trehalose alone as the cryoprotectant is about 65%, although ice formation (or opacity) is consistently observed when cooling these samples (data not shown). Of note, the average cell number per field of view was found to be almost the same for the three experimental conditions with very different cell viability (20–80%, see Figure 6), which indicates that dead cells are as recoverable as intact cells from the procedure for studying the immediate cell viability post vitrification.

Embryonic stem cells must be able to attach to a substrate in order to proliferate to their pluripotent state. Unlike the immediate viability, only a minimal number of cells were able to attach when using either 0.5 M trehalose (<2%) or 2 M 1,2-propanediol alone (~12%) as the cryoprotectant during vitrification (Figure 6). The attachment efficiency, however, was much higher (~72%) when using the combination of 0.5 M trehalose and 2 M 1,2-propanediol. Therefore, adding 0.5M trehalose in the 2M 1,2-propanediol solution can significantly improve the vitrification efficiency of the solution for mammalian cells, although trehalose is impermeable to the plasma membrane of mammalian cells and resides only in the extracellular space. This is probably because the addition of 0.5M trehalose further dehydrates the cells and stabilizes the extracellular solution against ice formation, both of which should improve vitrification of the mammalian cells [2,40,44].

The proliferation or growth of the attached ES cells post vitrification using the combination of 0.5 M trehalose and 2 M PROH was very similar to the proliferation of the control non-frozen samples. A robust increase in the number of cells per field of view was observed over a three day period for both the vitrified ES cells and the non-frozen samples (Figure 7A). This similarity in growth patterns is more evident in Figure 7B which shows the normalized number of cells per field of view calculated as the ratio of the cells per field of view at days 2 and 3 with respect to that of day 1 for each sample. The population doubling time for all cell groups was estimated to be approximately 21 hours based on a linear interpolation of the data. Therefore, the vitrification procedure did not appear to affect the growth characteristics of murine embryonic stem cells when they attached post vitrification.

The undifferentiated properties of the pluripotent ES cells were verified by the high levels of staining for the membrane surface glycoprotein SSEA-1 (Figure 8A) and expression of the green fluorescent transcription factor OCT-4 (Figure 8B). The merged view of the red (SSEA-1) and green (OCT-4) channel indicates extensive co-expression of the two markers and DAPI nuclei staining (blue, Figure 8C). Phase contrast image (Figure 8D) shows cells with high nuclei/cytoplasm ratios and compact colony formation typical of pluripotent ES cells. Histochemical staining shows strong expression for alkaline phosphatase at high magnification (Figure 8E) which was seen to be well distributed within each colony as observed at a lower magnification (Figure 8F). Collectively, the robust expression of the different markers that are characteristic to murine embryonic stem cells, along with proper ES cell morphology, suggests that the murine ES cells retained their undifferentiated properties as pluripotent cells post cryopreservation by vitrification. Of note, the method used to demonstrate that the cells are undifferentiated is qualitative and thus says little regarding the absolute level of expression of the chosen markers. Further studies to quantify the absolute level of expression of the markers and judge the capability of the ES cells to differentiate by present evidences such as embryoid body formation, immunocytochemistry, or differentiation markers are necessary to further verify the pluripotency of the cells post vitrification.

DISCUSSION

In this study, we developed a quartz microcapillary system to achieve ultra-fast cooling rates leading to vitrification at a low, nontoxic intracellular concentration of cryoprotectants (2 M). This new technique overcomes the critical problem associated with the traditional vitrification approach, with regards to using very high (generally more than 4M) and often toxic concentrations of cryoprotectants [13–15,36]. This study demonstrated that mammalian cells can be successfully cryopreserved by vitrification at a concentration that is nontoxic and close to concentrations used for traditional slow freezing protocols (1–2 M) [26,31]. Therefore, this ultra-fast vitrification cryopreservation technique combines the advantages of the traditional slow freezing and vitrification methods while it avoids their limitations. Of note, this new technique still requires storage at cryogenic temperature which makes it inconvenient in term of transportation, a disadvantage associated with all cryopreservation technique. Moreover, the open system of this technique in its current stage also raises issues related to contamination and regulation [22]. Developing an effective approach to seal the microcapillary at both ends that can withstand cryogenic temperature is necessary from both a commercial and clinical perspective.

Our solution studies indicate that 1,2-propanediol is a better cryoprotectant than ethylene glycol in terms of its capability for achieving apparent vitrification (i.e., no opacity). The concentration of 1,2-propanediol required for achieving apparent vitrification in the quartz microcapillary is as low as 1.9M. However, less than 20% of murine embryonic stem cells were able to survive and attach after cryopreservation using 2 M 1,2-propanediol alone as the cryoprotectant. Lack of opacity, therefore, is not a sufficient condition to guarantee viability

of the cells. This could be because the lack of opacity does not necessarily indicate that the sample has vitrified. The formation of ice micro-crystals can lead to a clear sample of crystalline ice. Alternatively, it may mean that vitrification itself is not a sufficient condition to ensure viability. Another possibility is that the cells were mainly damaged during the warming steps due to devitrification (i.e., ice formation of vitrified solution during warming), as critical warming rates to avoid devitrification are generally higher than critical cooling rates to achieve vitrification [11,13]. Devitrification is practically certain when using concentrations of cryoprotectants that barely vitrify during cooling. Based on these studies there is no way to tell which of these mechanisms was actually in action.

Our study also shows that extracellular trehalose and cell membrane permeating cryoprotectant 1,2-propanediol work synergistically to protect murine embryonic stem cells from cryo-injury during cryopreservation by vitrification. Although trehalose does not permeate the plasma membrane of the ES cells, adding 0.5 M trehalose to the vitrification solution can reduce the probability of ice nucleation and growth in the extracellular space. Furthermore, the increased extracellular solute concentration after adding 0.5 M trehalose should further dehydrate the cells and decrease the water activity both inside and outside the cells. As a result each cell in the vitrification solution resembles a single droplet of dehydration-concentrated biomacromolecules such as proteins with 2 M 1,2-propanediol, which could be vitrified more easily due to its high solute concentration and small volume [2,25,40]. Extracellular trehalose can also protect stem cells from cryoinjury by decreasing the probability of devitrification and/or recrystallization, one of the major injury mechanisms during the warming of cryopreserved samples [2,11,40,44]. The non-reducing disaccharide trehalose has also been shown to protect mammalian cells during cryopreservation by preventing cells from oxidative damage [1] and keeping cell membranes from coming in contact and fusing [7]. The ability of trehalose to protect cell membrane from damage during cryopreservation is evident in Figure 6, which shows that nearly 65% cells retained their membrane integrity post cryopreservation using 0.5 M trehalose alone as the cryoprotectant. Extensive ice formation was always observable after plunging the ES cell maintenance medium with 0.5 M trehalose into liquid nitrogen (data not shown), which suggests that trehalose could protect cell membranes from damage even in the presence of extracellular and probably intracellular ice. This observation is consistent with a recent study that shows that extracellular trehalose has significant protective effects in cryopreserving mammalian cells using the slow freezing approach[10,23,43], in which extracellular ice formation is inevitable.

Figure 6 shows the immediate cell viability based on membrane integrity is not much lower than the corresponding attachment efficiency for samples cryopreserved using 2 M 1,2-propanediol alone or the combination of 2 M 1,2-propanediol and 0.5 M trehalose. When using trehalose alone as the cryoprotectant, however, the attachment efficiency is much lower than the immediate viability assessed by cell membrane integrity. Therefore, caution should be taken when using membrane integrity assays to judge cell viability post cryopreservation and attachment efficiency is always recommended for judging the true cell viability post cryopreservation.

Since higher cooling rates lower the cryoprotectant concentration required to achieve vitrification [2,4,25,40], we are interested in further decreasing the amount of cryoprotectant required for vitrification via heat transfer enhancement either inside the cryopreservation solution or on the boundary between the microcapillary and the cryogenic fluid. Enhancing heat transfer inside the cryopreservation solution could be achieved by dispersing nanoparticles in the cryopreservation solutions to form nanofluids [27]. For example, studies have shown that adding a small amount of nanoparticles or nanotubes (< 1% v) into an aqueous solution can significantly increase (up to two-fold) the thermal conductivity of the solution [6,9]. Enhancement of the convective boiling heat transfer on the boundary could be achieved by

manufacturing microfins on the external surface of the capillary and using a different cryogenic medium such as subcooled liquid nitrogen (i.e., slush nitrogen) that has been shown to enhance the cooling effect in some studies [17,19,29,32,37,38], although the degree of heat transfer enhancement of slush nitrogen vs. liquid nitrogen for small (sub-millimeter) samples is not totally in accordance with each other in the literature [30]. Additionally, the enhancement of heat transfer during the warming step of a vitrification protocol is essential since critical warming rates to avoid devitrification are generally higher than critical cooling rates to achieve vitrification. The above mentioned approaches for the heat transfer enhancement during cooling should apply during warming as well. These studies are ongoing in our laboratory. Our ultimate goal is to further reduce the required cryoprotectant concentration to around 1M or lower.

SUMMARY AND CONCLUSIONS

In this study, we developed a novel ultra-fast vitrification approach for cryopreservation of sensitive mammalian cells using a small quartz microcapillary. The cryoprotectants used were 2 M 1,2-propanediol and 0.5 M extracellular trehalose. The intracellular concentration (2 M) of cryoprotectants is in the range of that used for slowing freezing. We characterized this new approach using thermal analysis, solution studies, and cell culture assays. Our thermal analysis indicates that using a microcapillary with an inner diameter less than 0.4 mm can significantly increase the cooling rate with respect to traditional straws. Our solution studies shows that 1,2-propanediol is a much better cryoprotectant than ethylene glycol in terms of its capability to achieve apparent vitrification. It was found that only 2 M 1,2-propanediol is sufficient to achieve apparent vitrification using the quartz microcapillary when plunging into liquid nitrogen. Our biological assays show that trehalose and 1,2-propanediol work synergistically to protect murine ES cells from cryo-injury during cryopreservation by ultra-fast cooling (vitrification). More than 70% of ES cells attached well, proliferated normally, and retained the undifferentiated properties of pluripotent cells post vitrification using the combination of 2 M 1,2-propanediol and 0.5 M trehalose. These results indicate that vitrification by ultra-fast cooling using a very low concentration of intracellular cryoprotectants (e.g., 2 M 1,2-propanediol) is a viable and effective approach for the cryopreservation of murine ES cells and potentially other important mammalian cells.

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Figure 1.

A comparison of the devices studied including the traditional straw (TS), the open pulled straw (OPS), and the so-called superfine open pulled straw (SOPS), and the proposed quartz micro-capillary (QMC). The proposed device has a diameter just slightly larger than that of a human oocyte (~150 μ m).

Figure 2. A schema of the physical problem for thermal analysis of the capillary and the grid design. The capillary was plunged into liquid nitrogen at a high speed of V ($>1\text{m/s}$).

Figure 3.

An image of the experimental setup used to determine the threshold cryoprotectant concentration required for complete avoidance of visible opacity (i.e., apparent vitrification) during ultra-fast cooling solutions in the quartz capillary. Opacity in the QMC was made visible by directing focused lights from two fiber optic lamps on the capillary and holding it against a dark background. Shown in the figure are two QMCs (held together using a tweezer at the bigger end) loaded with solutions of different concentrations of 1,2-propanediol after plunging into liquid nitrogen. The top one with 2 M 1,2-propanediol is clear while the bottom one with 1M 1,2-propanediol is opaque.

Figure 4.

Predicted average cooling rates from thermal analysis: (A) cooling rate as a function of the convective boiling heat transfer coefficient for the QMC, grid, OPS and TS; (B) cooling rates for quartz capillaries with various inner diameter and wall thickness; (C) cooling rates for plastic capillaries with various inner diameter and wall thickness; and (D) cooling rates of capillaries made of various materials with 10 μ m wall thickness and 180 μ m inner diameters (open dots are predicted data point and the dotted line is a simple trendline of the predicted data).

Figure 5.

The threshold concentration required for the complete absence of opacity (apparent vitrification) when cooling various CPA solutions in the traditional straw, open pulled straw (OPS), superfine open pulled straw (SOPS) and the quartz micro-capillary (QMC). Values represent the mean $\pm$ standard deviation of at least three different experiments.

Figure 6. Immediate cell viability and attachment efficiency of ES cells post cryopreservation using three different cryoprotectants and concentrations. The concentrations of trehalose and 1,2-propanediol (PROH) are 0.5 M and 2 M for all the conditions, respectively. Values represent the mean $\pm$ standard deviation of at least three different experiments.

Figure 7.

Proliferation of attached ES cells post vitrification: the absolute (A) and normalized (B) number of cells per field of view under a 10x objective for both control (non-frozen) and cryopreserved embryonic stem cells. The normalized number of cells per field of view was calculated as the ratio of the cells per field of view at days 2 and 3 with respect to that of day 1 for each sample. Values represent the mean $\pm$ standard deviation of at least three different experiments.

Figure 8.

Immunofluorescence and histochemical analysis for maintenance of undifferentiated properties of the pluripotent ES cells post vitrification. Fluorescence micrographs display high levels of staining for the surface glycoprotein SSEA-1 (A) and Oct-4 transcriptional activity as denoted by GFP expression (B). In panel C, the merged view of red and green channel indicates extensive co-expression of the two markers and DAPI nuclei staining (blue). Phase contrast image shows cells with a high nuclei/cytoplasm ratio and compact colony formation typical of pluripotent ES cells (D). Histochemical staining shows strong expression for alkaline phosphatase at 10x magnification (E) which was seen to be well distributed within each colony as observed at a lower 4x magnification (F). Scale bars, 100 μ m (C, also represents A and B), 200 μ m (E), 500 μ m (F).

Table 1

A list of the devices studied in the thermal analysis

Materials	OD, mm	ID, mm	Wall Thickness, mm
Traditional straw (TS, plastic)	2	1.7	0.15
Open pulled straw (OPS, plastic)	0.95	0.8	0.075
Grid (0.5 μ l, microdroplet)	1.24	N/A	N/A
Quartz micro-capillary (QMC)	0.2	0.18	0.01

OD: Outer diameter; ID: Inner diameter

Table 2

A list of the thermal properties used in the thermal model

Materials	Density, kg/m ³	Specific heat, J kg ⁻¹ K ⁻¹	Thermal Conductivity, W m ⁻¹ K ⁻¹
Solution	1022	3800	0.54
Plastics	1200	1500	0.2
Glass	2200	850	0.8
Quartz	2649	710	8
Stainless Steel	7817	460	16.3
Sapphire	3970	419	27.2
Gold	19300	129	320
Copper	8920	385	385
Silver	10490	232	406
Diamond	3500	502	1000

Table 3
A typical experimental matrix for the examination of apparent vitrification of the various cryoprotectant solutions in open pulled straw (OPS) and quartz micro-capillary (QMC). The single plus and minus symbols represent apparent vitrification and appearance of visible ice crystal (opacity) in the whole solution volume, respectively.

CPA Volume (%v)	12	14	16	18	20	22	24	26	28
Ethylene glycol (EG)	OPS	N/D	N/D	N/D	-	-/+	+	+	+
	QMC	-	-/+	-/+	+	N/D	N/D	N/D	N/D
EG: PROH (1:1)	OPS	N/D	N/D	-/+	-/+	+	+	+	N/D
	QMC	-/+	-/+	+	N/D	N/D	N/D	N/D	N/D
1,2-Propanediol (PROH)	OPS	N/D	N/D	-/+	-/+	+	+	+	N/D
	QMC	-/+	+	+	N/D	N/D	N/D	N/D	N/D

The symbol “-/+” indicates partial apparent vitrification and “N/D” represents “not determined”.

OPS: Open pulled straw; QMC: 0.2 mm OD Quartz micro-capillary