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Implications of storage and handling conditions on glass transition and potential devitrification of oocytes and embryos

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ABSTRACT

Devitrification, the process of crystallization of a formerly crystal-free, amorphous glass state, can lead to damage during the warming of cells. The objective of this study was to determine the glass transition temperature of a cryopreservation solution typically used in the vitrification, storage, and warming of mammalian oocytes and embryos using differential scanning calorimetry. A numerical model of the heat transfer process to analyze warming and devitrification thresholds for a common vitrification carrier (open-pulled straw) was conducted. The implications on specimen handling and storage inside the dewar in contact with nitrogen vapor phase at different temperatures were determined. The time required for initiation of devitrification of a vitrified sample was determined by mathematical modeling and compared with measured temperatures in the vapor phase of liquid nitrogen cryogenic dewars. Results indicated the glass transition ranged from −126 °C to −121 °C, and devitrification was initiated at −109 °C. Interestingly, samples entered rubbery state at −121 °C and therefore could potentially initiate devitrification above this value, with the consequent damaging effects to cell survival. Devitrification times were calculated considering an initial temperature of material immersed in liquid nitrogen (−196 °C), and two temperatures of liquid nitrogen vapors within the dewar (−50 °C and −70 °C) to which the sample could be exposed for a period of time, either during storage or upon its removal. The mathematical model indicated samples could reach glass transition temperatures and undergo devitrification in 30 seconds. Results of the present study indicate storage of vitrified oocytes and embryos in the liquid nitrogen vapor phase (as opposed to completely immersed in liquid nitrogen) poses the potential risk of devitrification. Because of the reduced time-handling period before samples reach critical rubbery and devitrification values, caution should be exercised when handling samples in vapor phase.

1. Introduction

Low temperature preservation of oocytes and embryos is a fundamental cornerstone of assisted reproductive technologies. Cryopreservation of reproductive cells has been traditionally achieved by slow cooling the samples at specific rates to allow cell dehydration [1,2]. However, the preservation outcome of oocytes and embryos by slow freezing equilibrium protocols is negatively affected by cryoinjury due to formation of intracellular and extracellular ice crystals, concentration of solutes during the

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freezing process, and prolonged cell exposures to toxic cryoprotectant and chilling temperatures [2–4].

Storage of cryopreserved reproductive cells is conducted in specialized cryogenic, thermally insulated vacuum flask dewars that hold cryogenic fluids such as liquid N_2 below their boiling point [5]. Smaller to medium-sized tanks (20–50 L) used by veterinary practitioners and in some laboratories are routinely filled with cryogenic fluid to maintain adequate chamber temperatures [6]. Although the recommendation is to maintain the dewars full at all times [5,7], manufacturers provide guideline static evaporation rates for individual models and suggest close monitoring of liquid N_2 levels based on specific usage conditions [7].

Cells stored in these containers are kept either immersed in liquid N_2 or in the immediate vapor phase [5]. Because the temperature of the vapor phase is not a constant (as opposed to liquid N_2, −196 °C), a lack of temperature homogeneity within the chamber is observed [8]. Noteworthy, storage recommendations for oocytes and embryos in vapor phase of liquid N_2 dewars were originally formulated for cells that had been cryopreserved using equilibrium, slow freezing protocols [5,6]. Cells cryopreserved under those conditions have been reported to undergo sufficient dehydration and minimal cytoplasm supercooling and thus are less likely to be damaged during warming. However, these recommendations may not be applicable to vitrified material, which has a higher risk of devitrification and can suffer irreversible cryoinjury.

Vitrification, the process of solidification of a sample into an amorphous, glassy state in absence of intracellular and extracellular ice crystals, requires high concentrations of cryoprotectants, extremely rapid cooling rates, and reduced volume handling. In the last decade, vitrification has progressively become the method of choice for the cryopreservation of human oocytes and embryos [9–11], and this trend is now being followed by veterinary and animal science practitioners for domestic and exotic animal species [12,13].

Devitrification is defined as the process of crystallization in a formerly crystal-free, amorphous glass solution [12,14–16]. Early experiments to study the warming behavior of vitrified aqueous solutions were conducted by Luyet [14–16] and Luyet and Rasmusen [17,18] using differential thermal analyses to detect enthalpy changes associated with transition events [17,18]. Unlike melting point, devitrification phenomenon has been described not as an individual point but as a temperature range determined by the composition of solution, presence of nucleating particles, among other factors [14,15,18–21].

The devitrification of the intracellular solution and the surrounding extracellular medium can lead to significant damage during the warming of cells [20,21]. Several authors have indicated that above the glass transition temperature (Tg) of the cytoplasm (approximately −120 °C to −130 °C), the vitrified cytoplasm of oocytes and embryos could enter a liquid transition, promoting devitrification and subsequent ice nucleation and crystallization [22–24]. There are limited reports on Tg of cryopreservation solutions [20–22], and those available are mostly water–sugar solutions and not the complex mixtures of balanced salt solutions, permeating and nonpermeating cryoprotectants used in current oocyte and embryo vitrification protocols [25–27].

To date, there are no reports on Tg of vitrification solutions used in the storage of oocytes and embryos. This information would be of value to calculate critical devitrification thresholds and to update recommendations for the storage of vitrified oocytes and embryos. Therefore, the objective of this study was to determine the Tg of a cryopreservation solution typically used in the vitrification, storage, and warming of mammalian oocytes and embryos. To analyze devitrification thresholds, a numerical modeling of heat transfer for a common vitrification carrier (open-pulled straw, OPS) was conducted. Finally, the implications of these results on specimen storage and handling conditions in N_2 vapor phase were discussed.

2. Materials and methods

2.1. Measurement of the Tg of the vitrification solution by differential scanning calorimetry

Current vitrification protocols require that cells be successively moved through increasing cryoprotectant concentrations (permeable and nonpermeable) before their vitrification by direct plunging into liquid N_2 and long-term storage. Therefore, the Tg of the final vitrification solution routinely used in our laboratory was determined by differential scanning calorimetry (DSC).

The Tg of the vitrification solution consisting of 2.8 M Me_2SO (Sigma D2650) + 3.6 M EG (Sigma 102466), and 0.65 M trehalose (Sigma T3663) in TCM199 (Invitrogen, USA 12350–039) with 10% vol/vol fetal bovine serum (Invitrogen 10100139) was measured using a differential scanning calorimeter (TA Instruments, New Castle, DE, USA) model Q100 controlled by a TA 5000 module with a quench cooling system under a N_2 atmosphere. Samples of vitrification solution were enclosed in sealed aluminum pans and quench cooled up to −150 °C. An empty pan was used as a reference sample. Pans were heated at 2 °C/min from −150 °C to 20 °C, with isothermal periods at the initial and final temperatures. Distilled water was also scanned using the same program to verify equipment calibration. The step change visualized in the heat flow curve as a function of temperature corresponds to a second order transition (Tg). In the present work, the midpoint temperature in the step curve of the thermogram was defined as Tg [28].

2.2. Mathematical modeling of devitrification thresholds

2.2.1. Numerical modeling of the warming process of OPS

The initiation of devitrification in vapor phase was analyzed conducting a mathematical modeling of devitrification thresholds for a commonly used vitrification support (OPS) loaded with vitrification solution.

The OPS consists of a French polypropylene straw pulled under heat to reduce its internal diameter, therefore minimizing the loading volume of solution. Open-pulled straw, and also other devices such as cryotop, cryoloop, cryotip, and so forth is a reduced volume because only the tip of the OPS is loaded with minimal volume of approximately 1 to 3 μL containing the oocytes/embryos by capillary action [12].

When the OPS is placed at a certain height over the liquid N_2, it begins warming, as the height increases the