ARTICLE IN PRESS

Cryobiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cryobiology

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

The effect of isochoric freezing on mammalian cells in an extracellular phosphate buffered solution

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ARTICLE INFO

Keywords:
Isochoric preservation
Hyperbaric
Mammalian cells
Madin-darby canine kidney epithelial cells

ABSTRACT

Isochoric (constant volume) freezing has been recently suggested as a new method for cell and organ preservation. As a first step in studying the effect of isochoric freezing on mammalian cells, Madin-Darby canine kidney epithelial cells (MDCK), were frozen in an isochoric system, in a simple extracellular phosphate buffered solution to −10 °C (96.5 MPa), - 15 °C (162 MPa) and −20 °C (205 MPa) for 60 and 120 min. Cell membrane integrity and cell metabolism were studied with a Live/Dead cell vitality assay and flow cytometry. We found that cell survival decreases with an increase in pressure (lower temperatures) and time of exposure. For example, 60% of cells survived 60 min at – 10 °C and only 18% survived 120 min at this temperature. Negligible survival was measured at – 20 °C. This study may serve as the baseline towards further research on techniques to optimize the effects of isochoric freezing on living biological matter.

Modern medicine relies heavily on long term, *ex vivo*, preservation of biological matter [2]. Motivated by acute medical needs, various techniques for biological matter preservation are being developed, including a family of techniques that employ high subzero °C temperatures for preservation [1]. Isochoric (constant volume) freezing, was suggested as a possible method for high subzero °C temperature preservation [7]. The basic considerations in isochoric preservation are shown in Fig. 1. In isochoric freezing, as the temperature of the system is lowered, the thermodynamic path follows the ice I - liquid water liquidus line (right hand insert in panel 1A) to the triple point. Panel 1A also shows the percentage of the system volume that will remain unfrozen at various temperatures to the triple point [7]. This has suggested that if the biological matter is placed in the part of the volume that will not freeze, damage from ice will be eliminated during isochoric freezing [7]. This is illustrated in the second insert in panel 1A. Thermodynamic analysis also shows that, the concentration of solutes in the unfrozen part of a freezing isochoric volume will be substantially lower than that in a system under atmospheric pressure [7], (Panel 1B). Therefore, isochoric freezing should also reduce solute damage during freezing, in comparison to freezing under atmospheric pressure. However, Panel 1C [5], shows that the pressure increases as the temperature is lowered towards the triple point. Elevated pressures are known to contribute to cell death, e.g., [3]. To the best of our knowledge all the studies on the effects of a combination of elevated pressure and subfreezing temperature were done in constant pressure, hyperbaric, systems, e.g., [3]. However, the thermodynamics of freezing in isochoric systems is different from the thermodynamics of freezing in a hyperbaric system; the former deals with minimization of the Helmholtz function and the latter with minimization of the Gibbs free energy which can lead to different processes during freezing [10]. Furthermore, the thermodynamic process path to subfreezing temperatures in isochoric freezing is different from the thermodynamic process path in hyperbaric systems.

The goal of this brief communication is to present first experimental results, designed to serve as a baseline for further research in the field, on the effects of temperature, pressure and preservation time during isochoric freezing of Madin-Darby canine kidney epithelial cells (MDCK) (MDCK, UCSF Cell Culture Facility, San Francisco, CA). The cells were grown in an incubator supplied with 5% CO_2 at 37 °C and were maintained in a flask in Dulbecco's Minimum Essential Medium (Invitrogen Corp., Carlsbad, CA), Earle's balanced salt solution supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% penicillin/streptomycin and 2 mM of L-glutamine. The cells were removed from the wall of the cell culture flask with a 0.25% Trypsin/EDTA (Invitrogen Corp., Carlsbad, CA) solution. For the experiments, the culture medium was replaced with Dulbecco's phosphate buffered saline (136 mM NaCl, 8 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, Invitrogen Corp., Carlsbad, CA). The cells were counted using a hemocytometer (Bright-Line, Fisher, Pittsburgh, PA) and their concentration adjusted to approximately 2.0×10^6 cells/ml in PBS. In each experiment repeat, four 2 ml polypropylene tubes (Fisherbrand, Pittsburgh, PA) were filled with the cell solution. A 1 mm radius circle

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https://doi.org/10.1016/j.cryobiol.2018.04.004
Received 25 October 2017; Received in revised form 6 April 2018; Accepted 6 April 2018

Please cite this article as: Preciado, J., Cryobiology (2018), https://doi.org/10.1016/j.cryobiol.2018.04.004

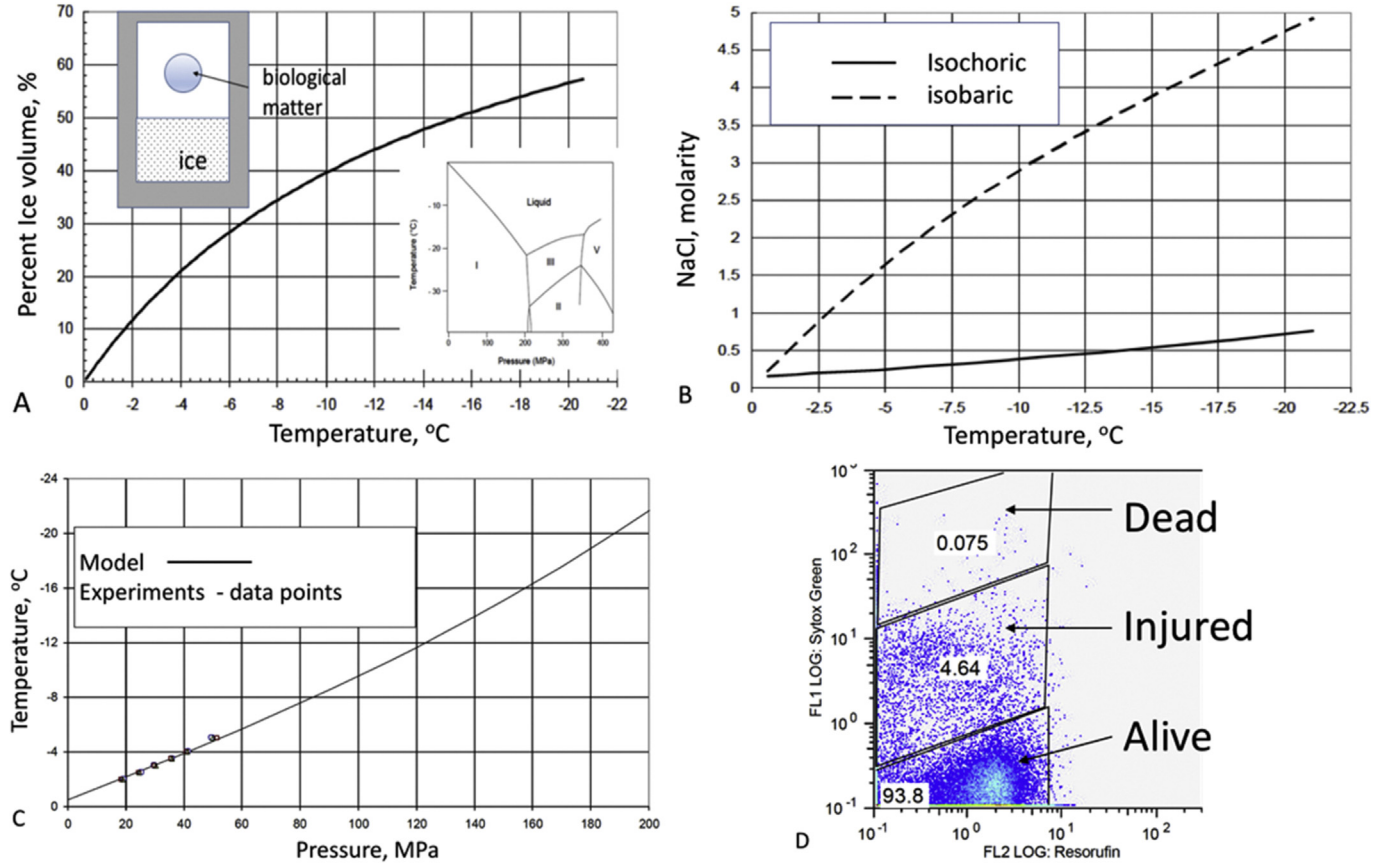

Fig. 1. A) Percentage volume that is ice, during the freezing of an isochoric system. Bottom insert - phase diagram. Top insert-isochoric chamber design [4] B) Molarity of NaCl as a function of temperature during the freezing of an isochoric system (straight line) and an isobaric system (dashed line) [7], C) The correlation between temperature and pressure during freezing in an isochoric system [7], D) Example of flow cytometry results for control MDCK cells, that remained in the incubator. The cells are stained with C_{12} Resazurin and SYTOX Green. The FlowJo program counts the number of cells in each gated area and outputs the percentage of the cells in each gated region.

was cut out of the top of the tube lid using a sharp blade to ensure that during the experiment there is no concentration difference between the interior and exterior of the solution. The isochoric freezing experimental system (pressure vessel) used in this study is identical to that described in Ref. [6]. Two of the tubes were placed in a nearly empty isochoric pressure vessel. Additional PBS is added to the core of the vessel, until the vessel is completely full and the vessel is closed. The tubes float to the top of the chamber, while freezing is initiated at the bottom of the chamber. The two remaining tubes are placed in the incubator as controls for the period of the experiment. In each experiment, the pressure vessel was placed in a temperature controlled bath, connected to the laptop and the temperature and pressure monitored as it approached the final set temperature of either −10 °C, −15 °C or −20 °C at an arbitrarily chosen cooling rate of 0.7 °C/min. Once both thermistors on the pressure vessel reached within one degree of the final set temperature the cold bath temperature was adjusted to maintain that final set temperature for an exposure time of 60 or 120 min. The system pressure and temperature were continuously monitored to ensure that the system was in equilibrium. At the end of the exposure period of either 60 min or 120 min, the isochoric pressure vessel was placed in a room temperature bath. Following the warming to approximately room temperature the contents of each tube were resuspended using a pipette and 1 ml of the resuspended cell sample was placed in a fresh polystyrene amber colored tube (Fisherbrand, Pittsburgh, PA). A total of six repeats was performed for each experimental condition. Dyes were added to each tube from a LIVE/DEAD Cell Vitality assay (Molecular Probes Inc., Eugene, OR) in order to determine metabolic activity and membrane permeability of the cells. SYTOX Green is a cell-impermeant, green-fluorescent nucleic acid stain that dyes necrotic or late apoptotic cells via their compromised plasma membranes. Using this assay, dead cells with compromised membranes will uptake the cell-impermeant, green fluorescent nucleic acid stain, SYTOX Green. C_{12}-resazurin is reduced to red-fluorescent C_{12}-resorufin in metabolically active cells. With these stains the dead cells will mostly emit green fluorescence and the healthy metabolically active cells will emit mostly red fluorescence. Injured cells will emit red fluorescence, though it will be decreased in comparison to healthy cells. Briefly, 20 µl of SYTOX Green nucleic acid stain was added to each 1 ml sample of cells. Ten microliters of C_{12}-resazurin dye is also added to that same 1 ml sample and then incubated at 37 °C and 5% CO_2 for 15 min. Following incubation, the tubes containing the dyed cell samples are placed on ice and transported to the UC Berkeley Cancer Research Laboratory Flow Cytometry Center. For the LIVE/DEAD Cell Vitality Assay the fluorescence is excited at a wavelength of 488 nm. The emitted light is measured at 530 nm and 575 nm. A graph with the y-axis representing the intensity of green fluorescence versus the x-axis representing the intensity of red fluorescence is created containing each data point and its respective fluorescence reading (Panel 1D). The data is analyzed using FlowJo (Tree Star, Inc., Ashland, OR), a flow cytometry analysis software program. Using a positive control for the live, metabolically active cells and a negative control for the cells that underwent necrosis (by exposure to hydrogen peroxide) it is possible to determine the fluorescence limits for these two regions. The FlowJo program creates a gate surrounding the green "dead" region and the red "alive" region (Panels 1D). The cells between the "live" and "dead" cell gates are defined as "injured". The FlowJo program also returns the