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The nematode *Caenorhabditis elegans* survives subfreezing temperatures in an isochoric system

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ABSTRACT

This study is the first experimental evidence showing that a living multicellular organism, the nematode *Caenorhabditis elegans*, can survive subfreezing temperatures in an isochoric (constant volume) thermodynamic system, while immersed in a simple isotonic solution, without the addition of cryoprotectants. Some of the test conditions were more extreme than those found at the ice/water interface of the Antarctic subglacial Vostok lake. On earth, life takes place in an isobaric (constant pressure) environment. In isobaric systems, subfreezing temperature survival of organisms in nature and subfreezing temperature preservation of living material for biotechnology and medicine, is made possible by use of cryoprotective chemicals additives. Our theoretical thermodynamic studies suggested that in an isochoric system, living biological material could survive subfreezing temperatures, without any cryoprotective chemicals. By confirming the theoretical predictions, this paper suggests a new technology for subfreezing preservation of cells, organs and organisms of possible value for biotechnology and medicine as well as new possible mechanisms of living organism survival in nature.

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1. Introduction

Cell and organ preservation for storage is important in biotechnology and medicine. It is used in diverse applications including biological procedures that require cell line storage and medical procedures that employ preserved cells and organs for transplantation [1]. Life processes are temperature-dependent, electro-chemical reactions; whose sum is the metabolism. Similar to the kinetics of every chemical reaction, the rate of metabolism is decreased by lowering the temperature. Most enzymes of normothermic animals show a 1.5–2 fold decrease in metabolic activity for every 10 °C decrease in temperature [2]. Therefore, lowering the temperature is how living cells and organs are preserved in

biotechnology and medicine [1]. The time of preservation increases with a decrease in preservation temperature. Many multicellular living organisms also lower their temperature to survive less than optimal environmental conditions; such as cold-blooded animals and hibernators in winter. However, life on earth takes place under atmospheric, isobaric (constant pressure), thermodynamic conditions. Under these conditions, water, which is the primary component of living matter, freezes at 0 °C when pure, and at –0.56 °C, in an isotonic concentration. Freezing completely disrupts life processes and living material cannot survive freezing, in their native state. In nature, organisms survive atmospheric, subfreezing conditions by generating chemical compounds that reduce the freezing temperature of the aqueous solution and modify the way in which the process of freezing occurs. Some of these compounds, such as antifreeze proteins, act in a non-colligative manner [3,4]. Others, such as glucose or glycerol act by colligative depression of the freezing temperature [5]. Cells [6,7] and organs [8,9] are preserved in a similar way, by artificially replacing part of the water in the cell with cryoprotective agents. Obviously the introduction of cryoprotective agents prior to preservation and their removal after preservation substantially complicates the preservation process [1].

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Motivated by an interest in finding improved methods for living biological matter preservation, we have developed the theoretical thermodynamics basis of isochoric (constant volume) preservation [10–12]. At subfreezing temperature, an aqueous isochoric system separates into two phases in thermodynamic equilibrium; ice I and a liquid phase. The separation in these two phases extends to the triple point between water, ice I and ice III which is at a temperature of 251.156 K and pressure of 209.9 MPa. Associated with this phase separation there is an increase in pressure. The phase diagram in the thermodynamic region of interest is shown in the insert in the right hand side panel of Fig. 1. Fig. 1 draws from our fundamental thermodynamic research [10–12], and shows the key elements that form the basis for our hypothesis that biological matter can survive subfreezing temperatures in an isochoric system. During slow freezing of biological matter with an isotonic composition, ice forms in the extracellular milieu first [1]. Since ice has a tight crystallographic structure and cannot contain any impurities, freezing rejects the solutes in the unfrozen phase and increases the solute concentration in the liquid phase [13]. Increase in intracellular and extracellular osmolality is the primary mechanism of cell damage during slow rate freezing [14,15]. Replacement of part of the cell water with a colligative compound (such as glycerol, ethylene glycol, or Me_2SO) reduces the increase in osmolality and is how cells survive slow freezing [14,15]. Addition and subsequent removal of cryoprotective agents is also how *C. elegans* are preserved [16]. We have found that in isochoric freezing, the osmolality of the unfrozen phase is much lower than that in isobaric freezing, at any subfreezing temperature to the triple point [10]. The theoretical results are shown in Fig. 1, the left hand side panel. We have also found that only a small fraction of the aqueous solution freezes to the triple point. The right hand side panel of Fig. 1, was derived from principles of thermodynamic equilibrium in an isochoric system with pure water [10]. It shows the percentage volume of ice in an isochoric system of pure water as a function of temperature. It is evident that at the triple point 43% of the volume is still unfrozen. In contrast, ice would occupy the entire volume at -0.001°C in an isobaric system at 1 atm Fig. 1, suggest that if biological materials are subjected to isochoric freezing temperature they will experience very low hyperosmotic concentrations, without the need for addition of colligative cryoprotectants. Furthermore, if kept in the unfrozen volume, they will not experience any ice. The results in Fig. 1 is the basis for our hypothesis that biological materials could survive subfreezing temperatures in

isochoric systems. Obviously, if this hypothesis is verified it may have important implications to cell, organs and organisms' preservation.

To test our hypothesis, we chose to perform experiments with the nematode *Caenorhabditis elegans*, a popular model organism in biological research [17]. An important attribute of *C. elegans*, discussed already in the pioneering paper of Brenner [16] is that they survive cryopreservation, freezing and thawing – which makes them a powerful tool for evolution (genetics) research. However, the cryopreservation process requires the addition of cryoprotective agents – as with all currently successfully cryopreserved living biological matter. Our choice of the *C. elegans* to explore the isochoric preservation hypothesis also draws from a study by Wergin et al. [18]. In that study, small, 10 μL droplets of solution containing the nematodes, were placed on glass microscope slides on a temperature controlled freeze plate at between -6°C to -8°C . Some of the droplets contained ice nucleating proteins, which ensured that the droplets froze. Other droplets, without the ice nucleating proteins did not freeze, because of the low probability of nucleation in this very small volume. After 2 min the temperature of the plate was raised to 24°C and viability was assessed by observing motion and transparency of the nematodes. Analysis has shown that 86.5% of the *C. elegans* survived in droplets that were supercooled for 2 min without freezing. However, 2 min of freezing reduced the survival to 2.5%. Obviously supercooling is not an effective way to cryopreserve organisms, because ice nucleation is a random statistical event, whose probability of occurrence increases with time. Freezing in isotonic solutions, under atmospheric isobaric pressure, kills the *C. elegans*.

The goal of our study was to examine if *C. elegans* can survive in the thermodynamically stable liquid phase of an isochoric system, during freezing.

2. Materials and methods

2.1. *Caenorhabditis elegans*

C. elegans worms were of the N2 wildtype strain and were obtained from the *Caenorhabditis* Genetic Center. Worms of mixed developmental stage were grown on standard nematode growth medium (NGM) plates with lawns of *Escherichia coli* bacteria (strain OP50) serving as food.

Fig. 1. Left panel. The increase in NaCl molality from an initial isotonic concentration, as a function of temperature, comparison between an isochoric and an isobaric system (Modified from Ref. [10]). Right panel. Relation between percentage volume of ice (IP%) and the phase transition temperature in an isochoric system of pure water, as a function of subfreezing temperature (Modified from Ref. [10]) (Insert – Temperature versus Pressure thermodynamic equilibrium phase diagram).

2.2. Experimental system

The experimental system is comprised of the instrumented isochoric chamber, a cooling bath, and the cryogenic vial with the nematodes. The isochoric chamber is described in greater detail in Ref. [12]. Photographs of the chamber components are shown in Fig. 2 (left panel). Briefly, it is based on a pressure vessel, (O-ring 316 SS, inner volume 100 ml, maximal pressure 241.3 MPa) custom designed by Parker Autoclave Engineers (Erie, PA, USA). The pressure inside the isochoric chamber is measured with an Ashcroft 4–20 mA Loop-Powered 20,000 psi Pressure gauge, connected through a NI myDAQ Connector (National Instruments) to a laptop and the data recorded and displayed with LabVIEW. A schematic of the isochoric system and experiment is shown in the top right hand side panel of Fig. 2.

The isochoric chamber is cooled in a cooling bath. We used two methods. Rapid cooling was achieved with a Nestlab RT-140 cooling system. Slow cooling was achieved by placing the isochoric cylinder in a slush of ice, water and salt in a composition designed to generate the desired temperature. We used a standard 12 mm inner diameter, 1.8 ml, Fisher Scientific cryogenic vial, capped and self-standing, as shown in Fig. 2 (bottom right hand side panel).

2.3. Experimental procedure

The samples were introduced in a cryogenic vial filled with an isotonic solution in such a way as to ensure that there was no air in the vial. The isochoric chamber was also filled with an isotonic solution. An ice nucleating surface was dropped to the bottom of the isochoric chamber to ensure that ice formation starts at the bottom of the chamber at a distance from the vials, which were on the top of the chamber. A small, 0.5 mm, hole was made in the wall of the chamber with the tip of a needle (Fig. 2), to ensure thermodynamic equilibrium between the interior of the vial and the interior of the isochoric chamber. Three vials were used in each experiment. The chamber was sealed, with care to avoid the entrapment of air bubbles. The schematic in Fig. 2 (top right panel)

illustrates the configuration in the isochoric chamber. The chamber was immersed in the cooling bath. The pressure was recorded and displayed in real time, using LabVIEW. We had no control over the cooling rate, except through the setting of the temperatures on the Nestlab device or in the slush solution. The experiment was terminated when the desired pressure was reached. The isochoric chamber was taken out of the bath and left to warm at room temperature. When the pressure reached atmospheric, the chamber was opened and the vials taken for viability evaluation.

Viability was determined by observing movement within an hour from cryopreservation and, when initial motility was marginal, a subsequent examination was taken 24 h later [18].

3. Results and discussion

Fig. 3 illustrates the criterion employed to evaluate post-isochoric cryopreservation survival. It consists in random selection of observation sites and counting worms that moved during the time of observation. Fig. 3 shows excerpts from a 10 s video recording of the worms under one microscope ocular site. The frames show adult worms and L2/L3 larvae. During the 10 s of observation all the *C. elegans* in the frame moved except the one marked with an arrow. In the different experiments some worms moved vigorously and others sluggish. In this study we evaluated only motion as a measure of viability. We observed that this measure of viability produced similar results immediately after the cryopreservation as well as 24 h after preservation. Of importance is the observation that sluggish motion did not correlate with death after 24 h. However, much more research is needed to evaluate the possibility of partial damage. Our criteria for viability here, was solely motion.

Fig. 4 shows the pressure-time history that the organisms experienced during the isochoric cryopreservation. The insert facilitates correlation between the pressure and the temperature in the two phase constant volume system. The insert also shows experimental results on the pressure-temperature correlations, that have verified our theoretical analysis [10]. Curve A, is for an

Fig. 2. Experimental system. Left panel, isochoric apparatus. Top right panel – schematic of the isochoric preservation system. Bottom right panel – the cryogenic vial with the pin making a hole.

Fig. 3. Images from a 10 s microscope video illustrating the way we evaluated viability. In this frame and time sequence only one L2 larvae (marked with an arrow) did not move. All the other adults and L2/L3 larvae moved, which we took as a measure for viability.

Fig. 4. Display of pressure versus time during different experiments. Insert: Temperature and pressure at thermodynamic equilibrium in a constant volume system containing ice and a solution, whose initial concentration is 0.9 mg/l NaCl.

experiment in which the pressure was elevated to 20 MPa (-2°C), in 10 min. The viability evaluation taken from five different sites showed the following live/dead ratio: adults (75/0), L2/L3 larvae (354/0). Obviously, all the nematodes survival brief isochoric exposure to -2°C . While, a depression of temperature of -2°C may appear small, any decrease in temperature is of importance in organ preservation. The normal temperature at which organs are

preserved for transplantation is 4°C (This is the temperature at which the organ will eventually find itself in an ice water mixture, because the lowest density of water occurs at 4°C). Many experiments have shown the value for preservation, of even a small temperature reduction. For example Amir et al., have shown that using antifreeze proteins to reduce the preservation temperature from 4°C to -1.3°C can extend the period of preservation of a heart

for transplantation from less than 6 h to 21 h [19], [20,21]. Okamoto et al. show that preservation of a lung in a supercooled state at -2°C , has substantial benefits over the preservation at 4°C [22]. Lungs stored using a method of lung preservation in which electric fields are facilitate supercooling, show better organ function after preservation at -2°C than conventional storage at 4°C . A recent paper has shown that liver can be transplanted after four days of preservation at -6°C with perfusion supercooling [23]. Conventional methods of liver preservation employ a temperature of 4°C , and livers can be preserved at that temperature for less than 8 h. Obviously lowering the storage temperature from 4°C to -6°C can substantially extend the period of preservation of organs for transplantation.

Curve B, is for an experiment in which the pressure was elevated to 45 MPa (-4°C), in about 20 min. The viability evaluation taken from three different test tubes showed that the overall live/dead ratio was 138/4 and the percentage live/dead in each of the tubes was (94.4%, 100%, 98.04%). The conditions of this experiment are particularly interesting in regards to life on earth under extremes of temperature and pressure. Thorough studies were performed on the thermodynamics at the bottom of the subglacial Antarctic lake Vostok [24]. The lake is under about 3.2 km–4.2 km of ice. The temperatures at different locations on the ice water interface in the lake range from -2.4°C to -3.15°C and the pressures of between 33.5 MPa and 43.9 MPa. Obviously the conditions that the nematode worm experienced in the experiment identified as curve B are more severe than those on the ice/water interface in lake Vostok. By wide extrapolation, this suggests that multicellular life could exist in the subglacial Antarctic lakes.

Curve C, is for an experiment in which the pressure was elevated to 65 MPa (-6°C), and the total excursion to and from that pressure took about 1 h (Fig. 4). Three test tubes with samples were used in this experiment. There were few adults in this series of experiments and the live/dead ratio was 15/6 (71% survival). The ratio of live/dead in L2/L3 larvae was less 65/108; (37% survival). The worms, while moving appeared to be more lethargic than in the previous experiments. However, the ratio live/dead was similar an hour after the experiment and 24 h after the experiment.

When comparing curves, A and C, it is obvious that the conditions in C were higher pressure and longer exposure to isochoric freezing. Therefore, in experiment D, we repeated pressure the pressure and temperature conditions from experiment A and exposed the nematodes to a length of exposure comparable to that in experiment C. To achieve these conditions, we immersed the isochoric chamber in a salt ice slush at -4°C , rather than use the Nestlab cooling apparatus. The results show that the survival of the worm was not affected by the longer exposure and it was similar to that in experiment A, 100% survival. This suggests that the organisms in experiment C succumbed to the higher pressure.

The goal of this study was to examine the survival of a multicellular organism during freezing in isochoric conditions. Survival was shown. However, there was no attempt to optimize conditions for preservation. The literature on hyperbaric preservation shows that the rate of pressure increase has an important effect on survival [25]. Lower rates of increase in pressure improve survival.

Obviously much research remains to be done if the goal is to use isochoric freezing for biological matter preservation. The effect of the rate of cooling during isochoric freezing is most likely an important parameter that should be examined next.

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