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Isochoric and isobaric freezing of fish muscle

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ABSTRACT

We have recently shown that, a living organism, which succumbs to freezing to −4 °C in an isobaric thermodynamic system (constant atmospheric pressure), can survive freezing to −4 °C in an isochoric thermodynamic system (constant volume). It is known that the mechanism of cell damage in an isobaric system is the freezing caused increase in extracellular osmolality, and, the consequent cell dehydration. An explanation for the observed survival during isochoric freezing is the thermodynamic modeling supported hypothesis that, in the isochoric frozen solution the extracellular osmolality is comparable to the cell intracellular osmolality. Therefore, cells in the isochoric frozen organism do not dehydrate, and the tissue maintains its morphological integrity. Comparing the histology of: a) fresh fish white muscle, b) fresh muscle frozen to −5 °C in an isobaric system and c) fresh muscle frozen to −5 °C I in an isochoric system, we find convincing evidence of the mechanism of cell dehydration during isobaric freezing. In contrast, the muscle tissue frozen to −5 °C in an isochoric system appears morphologically identical to fresh tissue, with no evidence of dehydration. This is the first experimental evidence in support of the hypothesis that in isochoric freezing there is no cellular dehydration and therefore the morphology of the frozen tissue remains intact.

1. Introduction

Long term *ex-vivo* preservation of biological materials, is of importance to numerous aspects of medicine, biotechnology, the emerging field of tissue engineering and the food industry. In fact, many applications, from the preservation of embryos to organ transplant, to preservation and transport of cell lines, to the world food economy depend on means for the preservation of biological matter, *ex-vivo*.

Life is comprised of a set of bio-electrochemical reactions known as metabolism. The rate of these metabolic chemical reactions is temperature dependent. Lower temperature slows metabolism in such a way that one decade of temperature reduction, in units of Kelvin, decreases the metabolism by a factor of between two and three [1]. This is the principle underlying the majority of biological matter preservation methods, currently used in medicine, biotechnology and food technology. When biological preservation of complex tissue requires functional survival, the preservation is hypothermic, i.e. above freezing temperatures. But, hypothermic temperature preservation is severely limited. Currently, hearts for transplantation can be stored at +4 °C for only 4–5 h and livers about 6–8 h. These periods of preservation are not sufficient, and attempts are made to extend the preservation periods [2].

In theory, reducing the temperature to absolute zero will stop all chemical reactions and facilitate unlimited preservation. However, under atmospheric pressure, physiological solutions freeze at −0.56 °C. This limits the temperature to which a biological system can be cooled without freezing. Freezing, the presence of ice and the associated changes in biological matter solution composition cause cell damage through additional mechanisms to those brought about by above freezing temperature, hypothermic, preservation [3,4]. Therefore, preservation by cooling is in a conundrum; preservation above freezing temperatures is limited in time while preservation below freezing temperatures induces additional

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severe damage, which makes storage of more complex organisms and tissues in a frozen state, yet, impossible.

Until now, most of the research on cold preservation of biological matter was done in isobaric, constant atmospheric pressure, for obvious reasons. Everyday life occurs under these conditions. However, recently, our group has taken a new approach to low temperature preservation and developed the fundamental thermodynamics of phase transformation of aqueous solutions in an isochoric, constant volume system [5–7]. The insert in Fig. 1 illustrates the difference in the thermodynamic path during an isobaric freezing process and an isochoric freezing process. The constant pressure, isobaric process is the vertical line in the insert. It is evident that when the temperature is reduced at constant pressure, the entire system freezes at the point the process line intersects with the liquidus line; for pure water at 1 Atm. pressure, this is 0 °C.

In isochoric freezing, the volume is constant, while the temperature is decreased. In a two-phase system, temperature and pressure are dependent properties, prescribed by the liquidus curve in the insert in Fig. 1. For pure water, the pressure and temperature at the triple point between ice I, ice III and liquid water are −21.985 °C and 209.9 MPa, respectively. Our thermodynamic analysis has verified experimentally and theoretically, that, the process path during the cooling of an isochoric system in the presence of an ice nucleating agent, is along the liquidus curve [5]. The thermodynamic analysis led to an interesting observation with relevance to biological matter preservation at subfreezing temperatures. The observation depicted in Fig. 1 shows the percentage of ice in the system as a function of temperature. It is of interest to notice that in an isochoric system, about 45% of the initial volume remains unfrozen, at the triple point. This has suggested a new concept for biological matter preservation. The concept is illustrated by the schematics in panel 2B. When a system is designed in such a way that the matter to be preserved occupies less than 45% of the total volume and nucleation is initiated outside the preserved volume, substantial amounts of biological matter can be preserved to the triple point temperature, without the biological matter actually freezing.

The first proof of the ability of an organism to survive under isochoric freezing was published in Ref. [8]. The study shows that a living invertebrate organism *C. elegans* survives exposure to isochoric freezing at −4 °C, but succumbs to atmospheric isobaric freezing to the same temperature [8]. The next step in our research is to study isochoric preservation in vertebrates. To this end, we use in this study fresh fish muscle tissue from Tilapia (Blue Tilapia: *Oreochromis aureus*). The study compares the histology of dorsal white muscle after, isochoric and isobaric freezing to −5 °C.

2. Materials and methods

2.1. Oreochromis aureus

In an effort to satisfy the 3R principles for animal care (Replacement, Reduction, Refinement) of Russell and Burch [9] our research group employs an experimental research model named "*vivens ex vivo*" [10]. Animal research is needed, because currently available methods for cell work and mathematical modeling cannot capture all the complexity of organized tissue. However, at an early stage of examining a hypothesis, it should be possible to gain insight into the value of a hypothesis without using a live animal for the experiment. Therefore, the "vivens *ex vivo*" concept is to use in a first examination of a hypothesis, fresh animal tissue whose provenance is animals which died from reasons not related to the study, such as other experiments or for food. In this study, we used fresh filets of fish (Blue Tilapia: *Oreochromis aureus*) prepared by a local fishmonger. The filets were white caudal muscle and the tissue was brought on ice, to our lab, in less than half an hour from preparation.

2.2. Isochoric system

The isochoric freezing system is a simple constant volume chamber, capable of withstanding the pressures that develop in the system, with minimal deformation. The chamber is instrumented with a pressure transducer. A photograph of the system is shown in Fig. 2C. The isochoric chamber is based on a modified stainless steel OC-1 pressure vessel, (O-ring 316 SS, inner volume 125 ml, working pressure 13,800 psi, test pressure 20,000 psi) custom designed by High Pressure Equipment Company (Erie, PA, USA). We used the standard O-ring made of BUNA-N, for sealing. The constant volume chamber is sealed with a screw and metal seal and is connected to an Ashcroft 4–20 mA Loop-Powered 20,000 psi Pressure gauge, connected through an NI myDAQ Connector (National Instruments, Austin TX) to a laptop and the data recorded and displayed with LabVIEW. For safety, a rupture disk limited the pressure to 60 MPa. The isochoric chamber was immersed in a water-ethylene glycol bath (50/50), cooled by means of a Nestlab RT-140 cooling system (Thermo Scientific, Waltham, MA).

2.3. Sample preparation

Fresh Tilapia fish (*Oreochromis aureus*), weight 500–700 g, purchased as fresh filets at a local fishmonger (Ranch 99, Berkeley), was used in this study. Cuboid dorsal muscle samples were cut from the filet and enclosed into cryogenic vials (standard 12 mm inner diameter, 1.8 ml, Fisher Scientific cryogenic vial, capped and self-standing) filled with a 0.9% saline solution [11], in such a way to ensure there was no air in the vials. We made a small hole (0.5 mm) in the vial's wall to ensure thermodynamic and osmolality equilibrium between the interior of the vial and the interior of the isochoric chamber.

2.4. Experimental protocol

The samples were exposed to −5 °C, for 3 h, under isobaric and isochoric conditions. The same chamber was used in the isobaric and isochoric experiments. Controls were cut from the fresh filets at the fishmonger site and immediately immersed in 10% neutral buffered formalin. The isochoric treatment was performed as follows. A metal 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. The isochoric chamber was filled with the 0.9% saline solution and sealed, with care to avoid the entrapment of air bubbles. Then, the chamber was completely immersed in the cooling bath and cooled to −5 °C. The pressure was monitored and recorded in real time, using LabVIEW. The isochoric chamber was warmed at room temperature until the pressure reached atmospheric. Then the chamber was opened to analysis the samples. The isobaric treatment followed the same procedure as the isochoric treatment except that the chamber was open to atmospheric pressure during the freezing. The samples were kept in the cooling bath at −5 °C for 3 h.

2.5. Histomorphology

After the treatments, all the samples were fixed with 10% neutral buffered formalin for 24 h and were later dehydrated in a series of ethyl alcohols of increasing concentrations (70, 85, 95, and 100%), cleared with xylene, and embedded in paraffin embedded wax (Shandon Excelsior Tissue Processor; Thermo Fisher Scientific,