RESEARCH ARTICLE | OCTOBER 18 2023

Liquid–solid equilibria and supercooling of Custodiol® in isochoric thermodynamic systems at subfreezing temperatures

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Physics of Fluids 35, 104107 (2023)
https://doi.org/10.1063/5.0169216

Physics of Fluids ARTICLE pubs.aip.org/aip/pof

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Cite as: Phys. Fluids **35**, 104107 (2023); doi: 10.1063/5.0169216
Submitted: 24 July 2023 · Accepted: 1 October 2023 ·
Published Online: 18 October 2023

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ABSTRACT

There is growing interest in using isochoric freezing and isochoric supercooling for the preservation of biological matter at subfreezing temperatures. Custodiol® is a commonly used intracellular composition type, subnormothermic preservation solution. It is anticipated that Custodiol® will also be used for isochoric freezing and isochoric supercooling preservation of biological matter. The thermodynamic properties of Custodiol® at subfreezing temperatures as well as the metastable behavior of the solution at subfreezing temperatures were not studied in the past. This study was designed to generate the thermodynamic data needed for the use of Custodiol® for the preservation of biological matter in isochoric systems at subfreezing temperatures. The experiments were performed in a specially designed isochoric chamber that can measure simultaneously the temperature and pressure in the isochoric chamber, and thereby correlate pressure and temperature at thermodynamic equilibrium in isochoric systems as well as the nucleation temperature in isochoric supercooling. The primary focus of this study is on determining the temperature at which nucleation is initiated and to identify the temperature threshold for nucleation due to its specific relevance to various applications in medicine.

INTRODUCTION

Organ transplantation is the sole treatment for end-stage organ failure.[1] One of the major challenges in organ transplantation procedures is the limited time that an organ can remain viable *ex-corporis* during transportation from the donor site to the recipient site.[2] While new methods for organ transportation are continually under development, the prevailing approach currently involves the use of the "static cooling" method. In this method, organs are placed in a bag containing a hypothermic preservation solution and transported while immersed in ice, maintaining temperatures between 0 and +4 °C. Several FDA-approved hypothermic preservation solutions are available for this purpose, each bearing commercial names such as Viaspan®, Custodial®, Euro-Collins®, and Celsior®.[3] These solutions typically exhibit an isotonic osmolality of ∼300 mOsm and often possess an intracellular ionic composition that is potassium-rich. Moreover, they incorporate various proprietary organic additives, resulting in colloid concentrations that range from 2 mm Hg to 30 mm Hg in different solutions.

While static cooling does prolong the ex-corporal survival of organs, this extension in survival time is still limited and often insufficient to meet the demands of most transplantation procedures. The "United Network for Organ Sharing" has provided guidelines on the permissible duration for organs to remain outside the body with this mode of preservation: hearts (4–6 h), lungs (4–8 h), liver (8–12 h), pancreas (12–18 h), intestines (8–16 h), and kidneys (24–36 h).[4] The underlying concept behind the subnormothermic mode of

preservation is as follows: uncontrolled metabolism is the mechanism by which extracorporeal biological matter deteriorates. Metabolism is temperature-dependent, so lowering the temperature reduces metabolism and extends the preservation time.[5,6]

Research is underway to find ways to prolong the ex-corporeal survival time of organs.[7] One apparent approach to extending the preservation period is by cooling biological matter to temperatures below the current preservation range of 0 to +4 °C, which further reduces metabolism. However, biological matter, primarily composed of water, will freeze below a certain phase transition temperature. It is well established that freezing damages biological matter.[8,9] Currently, research in this field focuses on developing solutions that allow preservation at sub-zero temperatures while preventing ice formation damage. A conventional cryopreservation technique involves modifying the solution's composition using chemicals known as cryoprotectants, such as glycerol.[9–13] These chemical additives depress the phase transition temperature colligatively and influence the mass transfer process across cell membranes to prevent freezing-related chemical damage. One drawback of these techniques is that in organ preservation, the organ must be perfused with these solutions before preservation.[2,14–18] The cryoprotective chemicals must enter the cells, and the cryoprotectants must be removed from the organ before transplantation. Additionally, cryoprotectants are toxic, and the perfusion and removal from biological organs pose challenges and can be detrimental to organ transplantation. In fact, while single cells can be preserved using these cryoprotectants, preserving larger volumes of living matter at sub-freezing temperatures remains a challenge.

Research in life sciences, including organ preservation, is typically conducted under isobaric (constant pressure) conditions, usually at atmospheric pressure. In an isobaric system where pressure remains constant, the entire system will freeze when the temperature of the solution falls below the intersection point between the liquidus line and the constant pressure line [as illustrated in Fig. 1(d)]. Modifying the composition, such as by introducing cryoprotectants, can alter and lower the freezing temperature. However, it is important to note that the system will still freeze entirely when it reaches the intersection between the liquidus line and the constant pressure line specific to the given solution.[1,24]

In our pursuit of developing new organ preservation technologies, we initiated a study on the thermodynamics of preserving biological matter at subfreezing temperatures within a constant volume system, known as an isochoric system.[19,20] In contrast to the isobaric freezing approach, isochoric systems exhibit a different behavior. In isochoric freezing, the process of freezing occurs along the liquidus line of water/ice thermodynamic equilibrium and results in a two-phase equilibrium (comprising ice and water) up to the triple point on the phase diagram [see Fig. 1(d)]. A simplified explanation for this phenomenon is linked to the change in density when water transforms into ice Ih. Ice Ih possesses a lower density than water. Consequently, in a constant volume system, lowering the temperature and the onset of freezing leads to an increase in pressure, and the system strives to attain equilibrium at this heightened pressure. Given that the system consists of ice and water in thermodynamic equilibrium, equilibrium at the elevated pressure can only occur along the liquidus line. Thus, the temperature-induced freezing in an isochoric system will unfailingly occur along the liquidus line, as depicted in Fig. 1(d). It is crucial to emphasize that in a constant volume system in thermodynamic equilibrium, the temperature and pressure of the two-phase system (comprising ice and water) are interdependent thermodynamic properties. Their relationship is delineated by the liquidus line in Fig. 1(d). In an isochoric system at a state along the liquidus line, the temperature of the system unequivocally determines the pressure, and vice versa. Consequently, measuring the pressure in the system can be employed to ascertain the temperature, utilizing the liquidus line in the phase diagram. This thermodynamic relationship serves as a foundational principle in our study.[5–7,10,11,15,21–23]

As indicated by the phase diagram in Fig. 1(d), the isochoric system comprises ice and water within a broad range of subfreezing temperatures extending to the triple point on the phase diagram, typically around −22 °C. Through thorough thermodynamic analysis, it has been demonstrated that, at any temperature up to the triple point, the volume of unfrozen water within the system remains substantial.[20] To illustrate, even at temperatures as low as −22 °C, which corresponds to the triple point, only about half of the system's volume is frozen, while the rest remains unfrozen. Based on this insight, a proposal emerged: in isochoric systems, biological matter can be positioned within the unfrozen portion of the system, allowing preservation without subjecting it to the detrimental effects of ice formation, even at subfreezing temperatures higher than the triple point.[19,20] A more sophisticated thermodynamic analysis of isochoric freezing, rooted in Helmholtz equilibrium principles, has been recently documented.[21,22] Experimental evidence supports these findings, demonstrating that whole organisms[23] and cellular structures like pancreatic islets[24] can endure preservation within the unfrozen portion of the isochoric system at subfreezing temperatures. Remarkably, this preservation method does not necessitate the use of any cryoprotective chemical additives. This simplifies the preservation process since there is no requirement for the infusion and subsequent removal of cryoprotective chemicals from the biological matter.

In our research on isochoric freezing, our group has made noteworthy discoveries regarding the stabilization of the supercooling state in aqueous solutions, both in the context of homogeneous nucleation[25] and heterogeneous nucleation.[26] A simplified explanation for this phenomenon is as follows: the random formation of an ice crystal nucleus momentarily elevates the pressure within the system, which then propagates through the fluid at the speed of sound to reach the rigid isochoric walls. This momentary pressure increase, in accordance with Le Chatelier's principle, works in conjunction with the surface tension effect resulting from the formation of an ice crystal nucleus. Together, they oppose the growth of randomly formed ice crystal nuclei, leading to the disassociation of these nuclei.

Another critical factor influencing ice nucleation in a supercooled fluid is cavitation. Any form of acoustic agitation can induce cavitation in the liquid, triggering ultra-rapid, high-pressure ice nucleation events.[27,28] The study of cavitation in liquids has garnered significant interest in fluid mechanics research.[29,30] Our research has demonstrated that isochoric confinement has the effect of suppressing cavitation-induced ice nucleation[31] (as detailed in Ref. 30). While experiments have provided empirical validation of the stabilizing impact of isochoric conditions on ice nucleation within supercooled systems,[32–34] it is important to acknowledge that much research remains to be undertaken to gain a comprehensive understanding of the underlying mechanisms.

As previously mentioned, various organ preservation solutions have been devised for temperatures above 0 °C. When designing

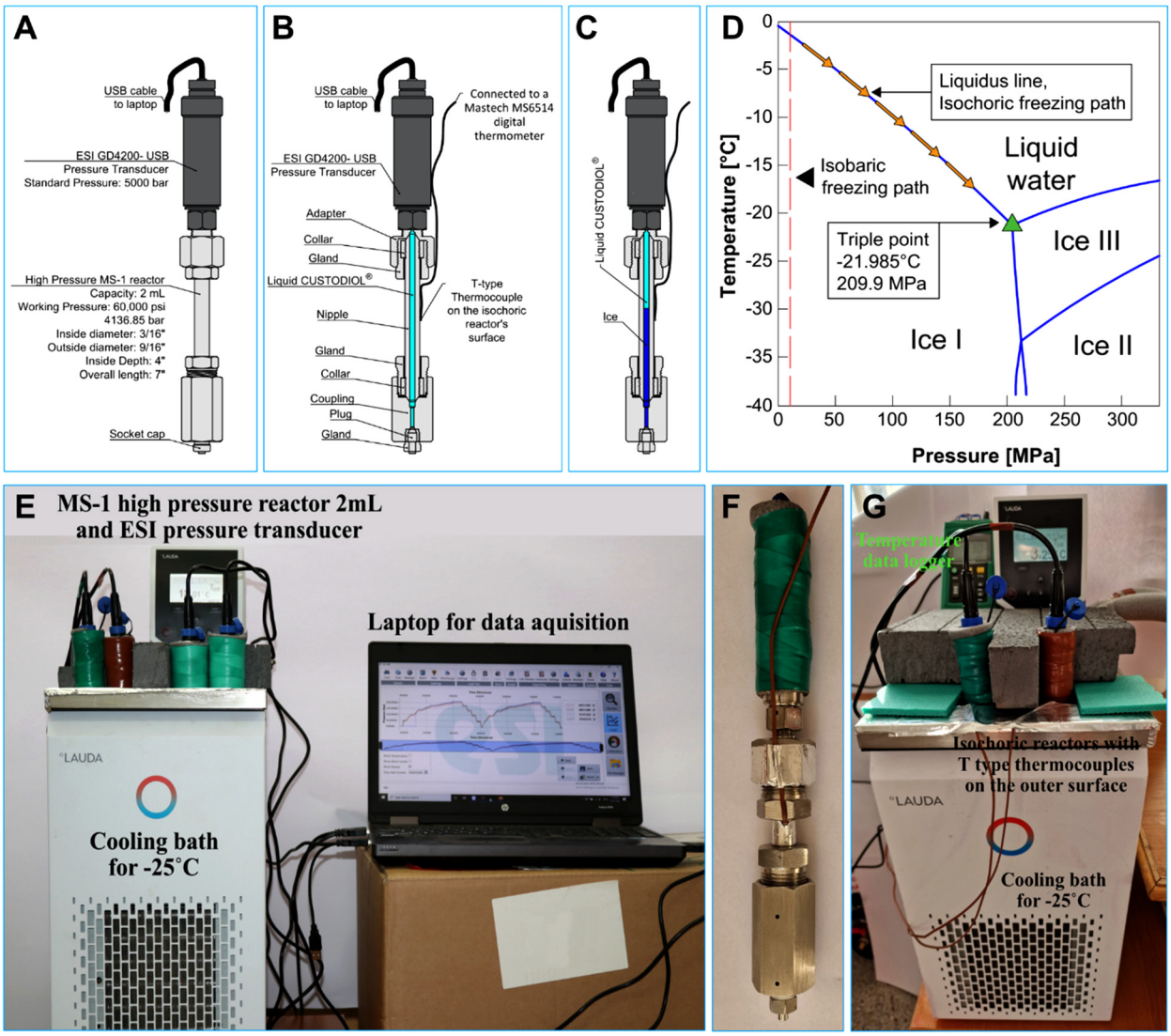

FIG. 1. (a) Figure of isochoric device consisting of the 316SS high-pressure 2 ml microreactor, socket cap, and the ESI GD4200-USB pressure transducer; (b) Figure of experimental isochoric reactor schematics in the initial state; (c) Figure of experimental isochoric reactor schematics while freezing; (d) The pressure–temperature phase diagram for water with the marking of the triple point;[27] (e) Photograph of the experimental system with the isochoric chambers in the cooling bath, side view; (f) Photograph of experimental isochoric reactor with the T-type thermocouple attached on the surface: (g) Photograph of the experimental system with the isochoric chambers in the cooling bath, the reactors with the thermocouples attached, and the temperature data logger, side view.

protocols for the preservation of biological matter using isochoric freezing or isochoric supercooling at subfreezing temperatures, it is imperative to have a thorough understanding of the phase diagram of these preservation solutions, along with insights into the stability of the supercooled state. Leveraging the unique characteristics of isochoric systems, where temperature and pressure are interdependent thermodynamic properties, and where pressure information propagates at a much faster rate than thermal diffusivity, we have developed a technique that enables us to construct the phase diagram of solutions relevant to isochoric freezing[35] and isochoric supercooling[32] (as discussed in Ref. 35). This technique involves simultaneous measurement of both temperature and pressure within the system. Our efforts have yielded valuable phase diagram information for several solutions of significance in the field of cryopreservation.[36–38]

The primary objective of this experimental study is to generate thermodynamic data concerning the freezing and supercooling processes in a Custodiol® solution within an isochoric chamber. Custodiol® initially designed as an intracellular crystalloid cardioplegic solution for myocardial protection during complex cardiac surgery is being used for organ preservation in transplant surgery[39,40] and has become a choice for isochoric freezing and isochoric supercooling preservation.[41] To carry out these experiments, we utilized a specially designed isochoric chamber equipped to simultaneously measure both temperature and pressure within the chamber. This unique capability

allowed us to establish correlations between pressure and temperature at thermodynamic equilibrium in isochoric systems, as well as determine the nucleation temperature during isochoric supercooling.[25,26,31–38,41]

MATERIALS AND METHODS

Materials

The study was done on a Custodiol® solution for infusion, produced by the German company Dr. Franz Kohler Chemie GMBH, Bensheim. Composition per 1000 ml: sodium chloride 0.8766 g, potassium chloride 0.6710 g, potassium hydrogen 2-ketoglutarate 0.1842 g, magnesium chloride × 6 H_2O 0.8132 g, histidine 27.9289 g, histidine × HCL × H_2O 3.7733 g, tryptophan 0.4085 g, mannitol 5.4651 g, calcium chloride × 2 H_2O. Osmolality 290 mosmol/kg.

In all pure water experiments, all identical micro-reactors were filled with pure distilled water (3 ml) (Distilled water, European Drinks SA, RO).

Isochoric chamber

The isochoric chamber used in this study is a metallic reactor made of 316 stainless steel, airtight and resistant to high pressures (up to 60 000 PSI or 413.69 MPa) Figs. 1(a)–1(c). The chamber deformation is negligible even at the upper limits of pressure and temperature which it is designed to withstand. The useful volume inside the chamber is 2 ml. This reactor is designed and manufactured in the USA by High-Pressure Equipment Company (Erie, PA, USA) and is the MS-1 model. Its dimensions are as follows: outer diameter 9/16 in., inner diameter 3/16 in., insight length of 4 in., and outside length of 7 in. A pressure transducer designed and manufactured by ESI Technology GD4200-USB in the United Kingdom for a range of 0–72 519 PSI, or 0–500 MPa, is used to measure the inside pressure. The pressure transducer is connected via ESI–USB Dynamic software to a laptop (HPProBook6 6570b) on which we visualize, store, and export data. The temperature is measured using two PerfectPrime TL0024 T-type thermocouples (2 m long, specific for high-accuracy measurements in the refrigeration and cryogenics field, with an excellent repeatability between −200 and 260 °C), connected to a Mastech MS6514 digital thermometer, which is connected to the same laptop to display and record temperatures. To limit the influence of ambient temperatures, we used polyethylene insulation for pipes, fixed with adhesive electrical tape to cover the pressure transducer. For safety, we recommend the use of a safety head, equipped with a ruptured disk at a pressure of 250 MPa.

Filling the chambers with Custodiol®

For the isochoric experiments, the isochoric chamber was loaded with the Custodiol® solution, at room temperature, taking care to avoid any air in the chamber, and capped. Avoiding air in the isochoric system is critical because air is more compressible than the solution. The presence of air affects the volume of ice that forms in the isochoric system and leads to erroneous pressure measurements.[42]

Cooling systems

One cooling system is a Lauda RE 1225 S (Germany) cooling device [Figs. 1(e) and 1(g)]. It has a constant temperature cooling bath, and it can maintain a constant set temperature. The cooling solution is 50% ethylene glycol, and the cooling device can maintain a constant set temperature in the bath filled with ethylene glycol at discrete values to −25 °C. For the experiments, the isochoric chambers were completely immersed in the ethylene glycol cooling bath with the pressure transducer protruding from the bath and insulated Fig. 1(d). Pressure and temperature in the chamber were continuously and simultaneously recorded throughout the experiment.

The second cooling system is a controlled rate cryogenic freezer from Planner, model Kryo 360, type 3.3 + MRV (Planer Limited, Middlesex, UK). The system is capable of maintaining constant temperatures from +40 to −180 °C and cooling or warming at a controlled rate. The equipment consists of the freezing chamber Kryo 360 Type 3.3, with a chamber volume of 3.3 Liters and a controller unit MRV. To achieve preservation temperatures down to −180 °C, the equipment needs a connection to a liquid nitrogen tank. We used a portable 50 Liters of liquid nitrogen cylinder Euro-Cyl from Chart (Chart Industries Inc, GA, USA), with a maximum allowable working pressure (MAWP) of 15 MPa (1.5 bar). A photograph of the system and its components is shown in Figs. 2(a) and 2(b). The isochoric chambers were placed in the cryogenic freezing in air [Fig. 2(b)].

Pressure–temperature at thermodynamic equilibrium

The goal of the first series of experiments was to measure the pressure in an isochoric chamber at thermodynamic equilibrium, at set temperatures, during the cooling and warming of the system. The experiment began by setting the temperature of the cooling bath to 0 °C. The temperature of the bath, with the isochoric chambers immersed in the bath, was lowered to a temperature of −25 °C, in increments of 5 °C. It was then increased to 0 °C also in increments of 5 °C. The temperature of the cooling bath was maintained constant at a preselected value until the pressure sensor and the temperature sensors show that the system is in thermodynamic equilibrium. The time for thermal equilibration was between 15 and 60 min. Six repeats were performed in this study.

Heterogeneous nucleation temperature

The first set of studies was performed with steady-state measurements in thermodynamic equilibrium. A second set of experiments was done to determine the heterogeneous nucleation temperature in an isochoric chamber during cooling with a constant cooling rate. For this set of experiments, we used the cryogenic freezer.

To detect the isochoric nucleation temperatures, the isochoric chambers were filled with the Custodiol® solution. The nucleation temperature in the isochoric chamber was determined by correlating the temperature measured on the outer surface of the chamber with the pressure.

RESULTS AND DISCUSSION

Pressure–temperature at thermodynamic equilibrium

The determination of thermal equilibrium was conducted through the following procedure. As the temperature of the solution surrounding the isochoric chamber was reduced or increased in steps of 5 °C, the solution inside the isochoric chamber began to undergo phase transformation. However, this freezing process unfolded slowly, primarily due to the intricate heat transfer dynamics both around and within the isochoric chamber. Factors such as the substantial thermal

FIG. 2. (a) Photograph of the experimental system with the isochoric setup using the cryogenic freezer; (b) Photograph of the isochoric reactors inside the controlled-rate cryogenic freezing chamber.

mass of the system and the enthalpy change associated with phase transformation within the isochoric chamber contributed to the prolonged nature of this process. As a result, achieving a state of thermal equilibrium within the system proved to be time-consuming. In contrast, the pressure within the isochoric chamber behaved hydrostatically, and any alterations in pressure propagated at the speed of sound. These pressure changes were directly linked to and induced by the freezing of water inside the isochoric chamber. Given the interdependence of pressure and temperature in an isochoric system, monitoring pressure changes served as an effective indicator of the thermodynamic state within the isochoric chamber. Specifically, when the pressure sensor ceased to detect any further fluctuations in pressure, it signified the attainment of thermodynamic equilibrium within the system. At this stage, it was confirmed that the temperature and pressure identified a state of thermodynamic equilibrium along the liquidus line. Typically, the time required for the system to reach this state of thermodynamic equilibrium fell within the range of 15–60 min. This is how the data in Fig. 3 were gathered.

Figure 3 displays pressure as a function of time in two of the six replicates. The temperature was changed stepwise, and the constant temperature values used in this set of experiments are listed on the horizontal lines in the figure. Each temperature was kept constant for 15 min, except for the −25 °C case, when the temperature was kept constant for 50 min.

The results should be read as follows: the intersection between the pressure–time curve and the horizontal temperature lines delineates regions of constant temperatures. For example, during cooling, the part of the pressure–time curve between the −10 °C and the −15 °C horizontal lines represents the pressure temperature correlation for the period when the temperature of the bath was lowered from −10 to −15 °C. However, during heating, the part of the pressure–time curve between the −10 and the −15 °C is for the period in which the temperature of the bath was increased from −15 to −10 °C. The figure also lists, next to the constant temperature line, the pressures recorded at that temperature at steady state and the standard deviation from six measurements—three/four during cooling and five during warming.

Several interesting observations emerge from this figure. First, it is evident that steady-state values of pressure are achieved after 15 min

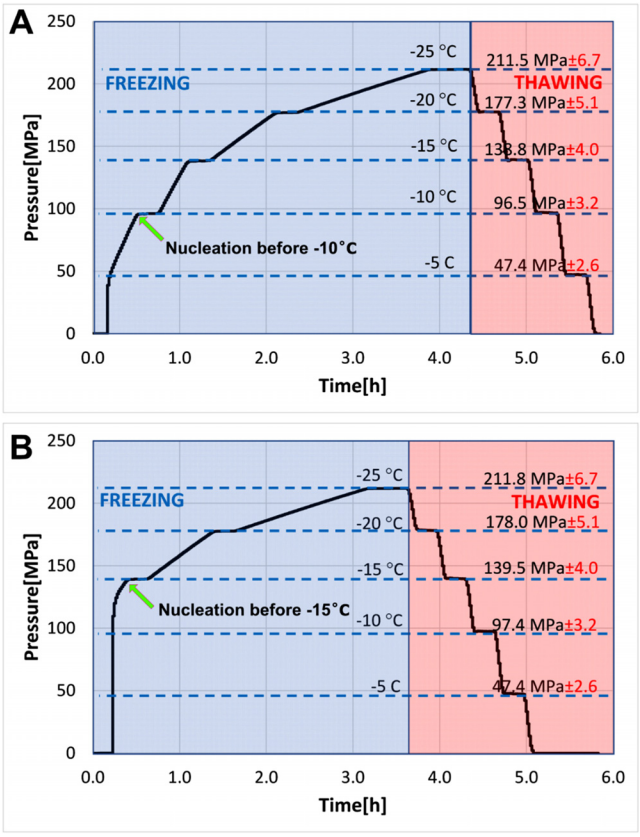

FIG. 3. Typical results obtained for Custodiol®, in isochoric (constant volume) conditions, using the cooling bath. Six repeats were performed, and the results are typical for all six replicates. Pressure as a function of time during cooling and warming in an experiment with nucleation at a nucleation temperature below −10 °C (a) and with nucleation below −15 °C (b). The experiments were performed by setting a constant temperature for periods of 15 min during cooling and warming and verifying that the pressure remains constant during this period. The measured constant temperatures and their corresponding steady-state pressure (with the standard deviation) are listed on the figure.

TABLE I. Temperature–pressure correlation for Custodiol® in isochoric systems.

Custodiol®	Process	Pressure at −5 °C (MPa)	Pressure at −10 °C (MPa)	Pressure at −15 °C (MPa)	Pressure at −20 °C (MPa)	Pressure at −25 °C (MPa)	Nucleation temperature (°C)
Experiment 1	Freezing			138.3	176.9	211.3	−11.708
	Thawing	47.0	96.8	139.0	177.4	211.3	
Experiment 2	Freezing			139.2	177.7	211.8	−12.957
	Thawing	47.4	97.4	139.8	178.2	211.8	
Experiment 3	Freezing			139.3	177.8	212.2	−13.077
	Thawing	47.8	97.6	140.1	178.6	212.2	
Experiment 4	Freezing		97.7	139.6	177.4	211.0	−9.851
	Thawing	48.9	98.0	140.2	178.0	211.0	
Experiment 5	Freezing			139.1	177.2	211.4	−11.734
	Thawing	48.1	97.0	139.5	177.7	211.4	
Experiment 6	Freezing			139.9	178.1	212.0	−12.33
	Thawing	48.4	97.9	140.3	178.5	212.0	

at any given constant temperature. The other interesting observation is that the same values of pressure were obtained at the specified constant temperatures both while cooling and while heating to those temperatures; i.e., there was no hysteresis. This suggests that, in this study, the measured values of pressure and temperature were at thermodynamic equilibrium. There is no data point for −5 °C during freezing in all experiments. This is because the system supercooled and freezing by random nucleation occurred only at a temperature lower than −5 °C. The results from this study are assembled in Table I, to aid in the interpretation of the results.

It is interesting to notice that the ice nucleation temperature listed in Table I varies over a range of temperatures between −9.85 and −13.08 °C. This is expected, as nucleation is a stochastic process, and even under controlled conditions, there can be some inherent variability. Additionally, nucleation is often sensitive to very small changes in the purity of the solution, cooling and warming rates, and chamber wall surface. In general, it was found that while the nucleation temperature is a stochastic value, the maximal range of nucleation temperatures is consistent, repeatable, and predictable for the same experimental system.[43]

Heterogeneous nucleation temperature

In Fig. 4, our experimental setup was configured to directly attain and stabilize at −20 °C within the cryogenic freezer, commencing from room temperature. As clearly depicted in the plot, the system successfully achieved thermodynamic equilibrium, which occurred during the time interval spanning from minute 82 to minute 135. It is important to note that the temperature shown in the plot corresponds to the temperature within the cryogenic freezer's chamber, as measured using a thermocouple affixed to the exterior surface of the isochoric chamber. This measurement, however, does not represent the

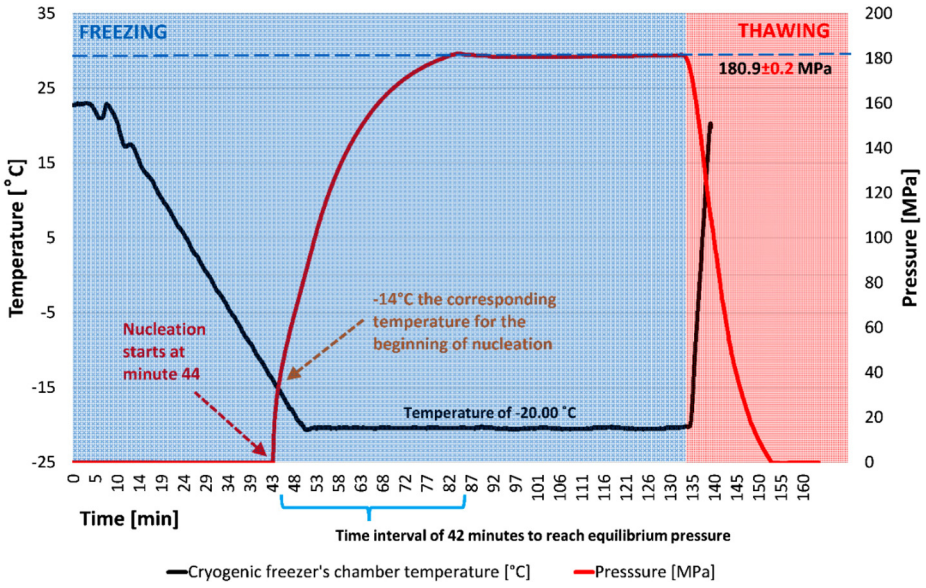

FIG. 4. Measurements of temperature and pressure during cooling to identify the isochoric nucleation temperature.

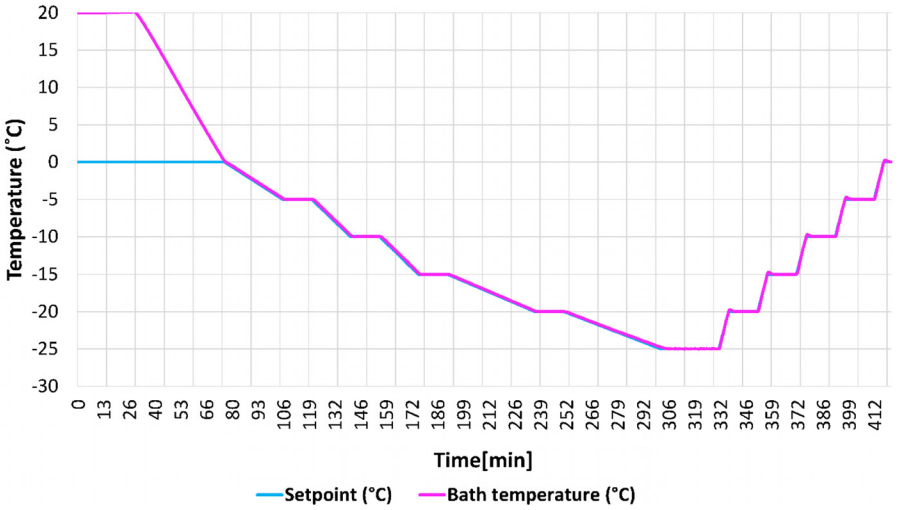

FIG. 5. Cooling bath protocol used in water experiments.

temperature of the liquid contained within the isochoric chamber. Figure 4 shows the pressure trace during cooling and the temperature trace. The point in time at which the pressure begins to increase indicates ice nucleation. We consider the heterogeneous isochoric nucleation temperature as the temperature at this time. The experimental results from six repeats are also tabulated in Table I.

A separate series of measurements was conducted to determine the heterogeneous nucleation temperature of distilled water within an isochoric chamber during the cooling process using the cooling bath system. The cooling protocol for distilled water followed the same procedure as that employed for Custodiol®, and the details are illustrated in Fig. 5. Each experiment commenced from ambient temperature, with the initial set point established at 0°. Subsequently, we incrementally lowered the temperature in 5 °C intervals until reaching −25 °C. At each temperature threshold (which was set at multiples of 5°), we maintained the system in equilibrium for a duration of 15 min, with the exception of the −25 °C threshold, where it remained in equilibrium for 20 min. Upon returning to 0 °C, we also allowed for a 15-min equilibrium period at each threshold.

Following this protocol, the typical results obtained for distilled water, in isochoric (constant volume) conditions, using the cooling bath are presented in Fig. 6. These results are consistent with similar values observed in Ref. 37. To replicate the Custodiol® experiments in the cooling bath system, six repeats were performed for distilled water too, and the results are typical for all six replicates. A comparison between the isochoric heterogeneous nucleation temperature for Custodiol® and pure water is given in Table II.

Table II is a comparison between the temperature/pressure correlation, i.e., the liquidus line, in Custodiol® and pure water. There is a statistically significant difference between these two substances. The p value is 0.815 if we compare the two substances with a Tukey's test (Minitab® version 20.4, Minitab, LLC, PA, USA). The difference can be attributed to the various additives that are incorporated in the Custodiol® solution (see Materials section). Interestingly, the freezing point depression from the various additives to the Custodiol® solution is substantially higher than what would be expected from the osmolality of the solution (about 300 mOsm). This should be about −0.6 °C. The difference can be explained by the nature of ice crystals and the

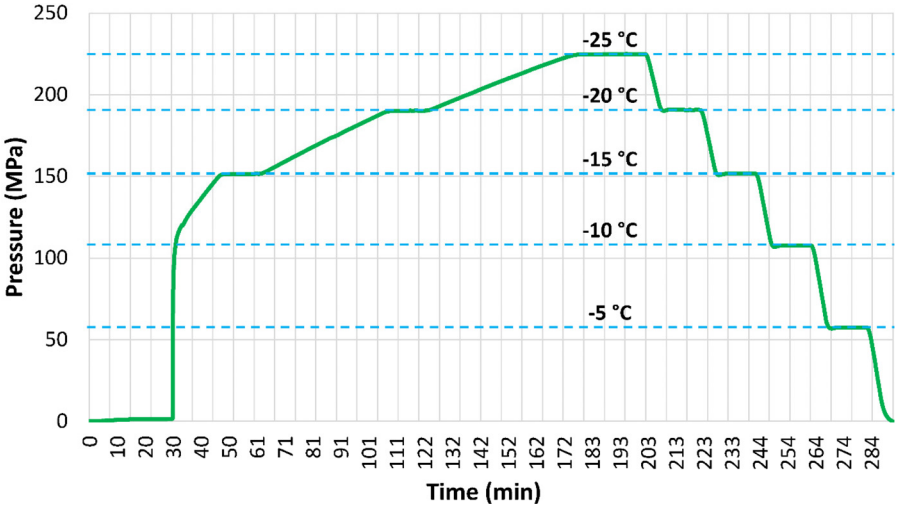

FIG. 6. Typical results obtained for distilled water, in isochoric (constant volume) conditions, using the cooling bath. Six repeats were performed, and the results are typical for all six replicates.

TABLE II. Temperature–pressure correlation for Custodiol® and water in isochoric systems. The pressures and the nucleation temperatures are given as average values ± standard deviation.

Substance	Pressure at −5 °C (MPa)	Pressure at −10 °C (MPa)	Pressure at −15 °C (MPa)	Pressure at −20 °C (MPa)	Pressure at −25 °C (MPa)	Average nucleation temperature (°C)
Custodiol®	47.9 ± 0.69	97.5 ± 0.45	139.5 ± 0.58	177.8 ± 0.52	211.6 ± 0.44	−11.94 ± 1.18
Water	57.3 ± 0.30	107.0 ± 0.65	150.9 ± 1.56	189.0 ± 2.46	219.5 ± 5.73	−10.88 ± 2.01

process of freezing in an isochoric chamber.[20] Ice crystals have a tight crystallographic structure and cannot incorporate any solutes. Therefore, in an isochoric system, when ice forms, the solutes in the frozen volume are ejected into the unfrozen solution and contribute to the increase in concentration in that solution. This leads to the further depression of the freezing temperature. The results of these experiments illustrate the need for studies of the kind done in this study, for every preservation solution. This type of data is imperative in the design of optimal isochoric cryopreservation protocols, because it provides information on the chemical environment that the biological matter will encounter during isochoric cryopreservation. It is possible that the increase in solute concentration may become toxic by itself and this will limit the subfreezing temperature in which an organ can be safely preserved.

Another interesting observation from Table II is that while the pressure/temperature correlation is very different between pure water and Custodiol®, the nucleation temperature is similar. A possible explanation is that when both solutions nucleate, the composition is mainly water. Only after the onset of freezing, there is the rejection of the solutes from the volume occupied by ice into the unfrozen portion, which affects the concentration of solutes.

The chambers were instrumented with thermocouples on the outer surface of the chamber at the same location in each, as shown in Figs. 1(b), 1(c), and 1(f). The onset of nucleation is marked by the recalescence (a sudden spike in temperature) that accompanies crystallization in supercooled liquids, which was monitored via thermocouple for all chambers and can be observed in Fig. 7. Additionally, because isochoric freezing yields only partial transition of the system to ice, and thus produces weaker recalescence signal, nucleation under isochoric conditions is indicated by the detection of a sudden increase in pressure, as can be observed in Figs. 3, 4, and 6.

Statistical analysis of these data to compare the heterogeneous nucleation temperature for Custodiol in comparison with pure water was done by one-way ANOVA, with Tukey's test used for the multiple comparisons test. Figure 8 shows the box plots. The statistical analysis demonstrates that the nucleation temperature for Custodial and pure water under isochoric conditions are almost the same. It should be emphasized that the nucleation temperature is for this device. A more general analysis of the probability for nucleation can be found in Ref. 29.

CONCLUSIONS

An experimental study was performed, which reports the temperature/pressure correlation in a commercial hypothermic solution for organ preservation, Custodiol®. To the best of our knowledge, this is the first study that investigates supercooling and the nucleation behavior of Custodiol® in isochoric conditions. The data can be used to predict the temperature/pressure correlation during isochoric freezing. It was interesting to find that the liquidus line of the preservation solution does not follow the pure water liquidus line. This is because

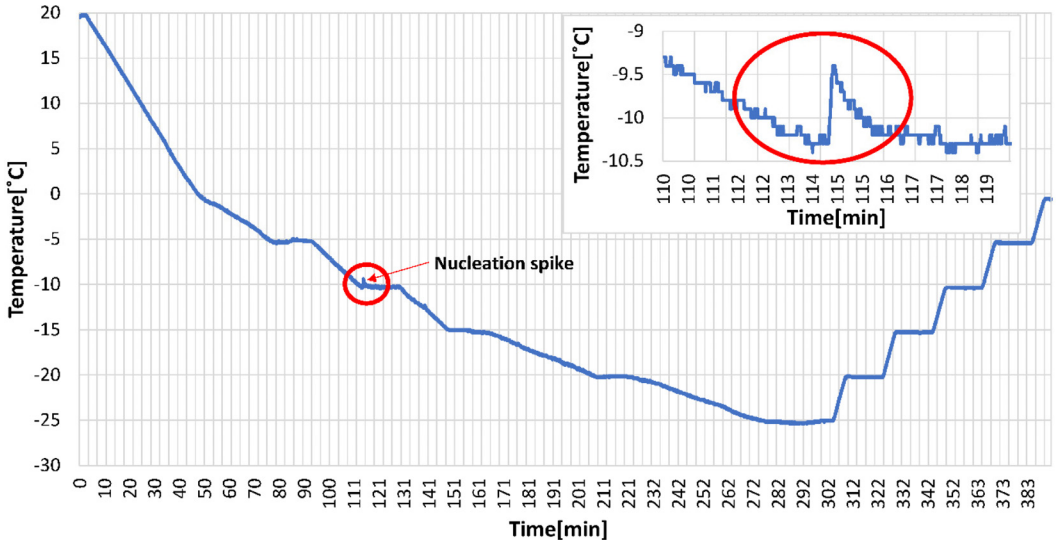

FIG. 7. Isochoric reactor's surface temperature measurements to identify the isochoric nucleation temperature in water experiments.

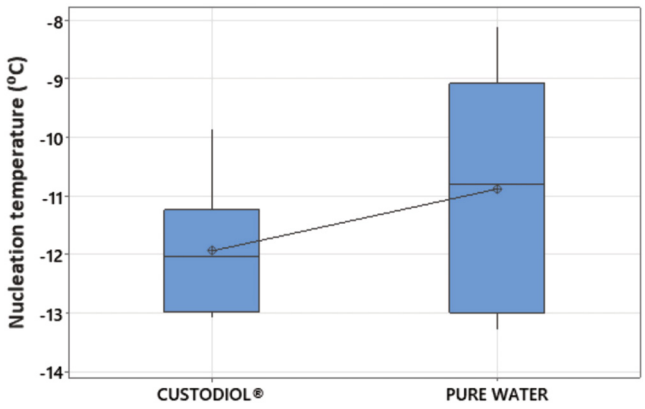

FIG. 8. The boxplots for Custodiol's nucleation temperature vs pure water nucleation temperature in isochoric conditions.

ice cannot contain any solutes, and therefore, the composition of the liquid part of the system also changes with temperature, as part of the isochoric system is replaced by pure water in the form of ice. This suggests the need for studying the pressure/temperature correlation in every preservation solution before preservation by isochoric freezing, because higher concentrations of some solutes could be toxic.

It was also interesting to find that the ice nucleation temperature is similar in water and Custodiol®. This suggests that isochoric preservation in a supercooled system may be independent of the composition of the preservation solutions. While this must be confirmed with other preservation solutions, this can aid in the design of isochoric supercooling protocols, by removing the concern over the effect of the actual composition of the preservation solution on the ice nucleation.

ACKNOWLEDGMENTS

This work was supported by a grant of the Romanian Ministry of Education and Research, CNCS–UEFISCDI, Project No. PN-III-P4-ID-PCE-2020-1706, within PNCDI III.

This work was supported by a grant of the Romanian Ministry of Education and Research, CCCDI–UEFISCDI, Project No. PN-III-P2–2.1-PED-2019-5409, within PNCDI III.

AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Author Contributions

Ștefan Ioan Câmpean and George Andrei Beșchea are co-first authors.

Stefan Ioan Campean: Conceptualization (equal); Formal analysis (equal); Investigation (equal). **George Andrei Beschea:** Conceptualization (equal); Formal analysis (equal); Investigation (equal). **Maria Bianca Tabacaru:** Data curation (equal); Validation (equal). **Alexandru Serban:** Resources (equal); Validation (equal). **Irinel Popescu:** Supervision (equal); Validation (equal). **Florin Botea:** Validation (equal). **Boris Rubinsky:** Supervision (lead); Writing – original draft (lead). **Gabriel Nastase:** Conceptualization (supporting); Formal analysis (supporting); Funding acquisition (lead); Investigation (supporting); Project administration (lead); Supervision (supporting).

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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