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PERSPECTIVE

A STATE OF THE ART REVIEW OF ISOCHORIC CRYOPRESERVATION AND CRYOPROTECTANTS

George-Andrei Beșchea[1*+], Ștefan-Ioan Câmpean [1+], Maria-Bianca Tăbăcaru [1], Beatrice-Georgiana Vuțoiu [1], Alexandru Șerban [1] and Gabriel Năstase [2]

[1] Transilvania University of Brașov, Faculty of Civil Engineering, Department of Building Services, Brașov, Romania.
[2] University Politehnica of Bucharest, Faculty of Mechanical Engineering and Mechatornics, Thermotechnics, engines, thermal and refrigeration equipment Department, Bucharest, Romania
*Corresponding author's E-mail: george.beschea@unitbv.ro
+Co-first authors

ABSTRACT

There is a developing enthusiasm for discovering new methods, cryoprotectants, systems and devices for cells, tissues, and organ preservation in medicine, in sub-zero temperature conditions and a growing interest in developing more efficient and economical methods for long-term preservation of food in a frozen state. Most of the preservation protocols currently used in medicine and food preservation involve the use of atmospheric pressure, and temperatures lower than normal body temperature in medicine, or lower than room temperature in the food industry. In this state of the art review, we analyzed the results of a new preservation method that uses an isochoric system. We aimed to offer a clear overview of the potential of this new technology. Firstly, to study the origins of isochoric preservation, we searched using the WoS Database. A search with the world "isochoric" returned 488 results. A more specific search of the term "isochoric freezing" returned 94 results. From these searches, we selected the 12 most relevant articles and discuss them here in detail. We present an overall characterization and criticism of the current use and potential of this new preservation method that can be used in the medicine and food industry. The main findings indicate encouraging results for the tested biological matter, including for the preservation of food products (e.g. cherries, spinach, potatoes), biological organisms (e.g. *Caenorhabditis elegans*, *Escherichia coli*, *Listeria*, *Salmonella typhimurium*), organs (e.g. rat hearts), tissues (e.g., tilapia fish filets) or cells (e.g., mammalian cells, pancreatic cells). Accordingly, we conclude that the isochoric system holds huge potential as a new technique in the field of preservation.

Keywords: cryopreservation; cryoprotectants; historical events; isochoric systems.

INTRODUCTION

Artificial cooling is part of our species' concerns to create better living conditions (1). The preservation of various biological materials dates back to ancient times since perfectly preserved animal bodies (especially mammoths) have been found in the Arctic Zone (2). In ancient China, beginning in the 11th century, during the cold period of the year, locals collected snow and ice that they stored in cellars. It was then used during the warm period of the year for cooling spaces and food (1). Similar procedures have also been described in ancient Egypt (approximately 2500 BC), in India or during the Roman Empire.

Beyond these early and rudimentary discoveries of the use of artificial cold for various purposes, it is appropriate to mention that the true momentum of artificial cooling was achieved with the discovery of refrigeration mixtures in the 16th to the 17th centuries (1) and the principles of thermodynamics in the 19th century. Since then, the cold industry has experienced rapid evolution. There have been many discoveries worth mentioning, such as the discovery of mechanical compression refrigeration installations by Jacob Perkins in 1834 called "Apparatus and means for producing ice, and in cooling fluids"(3) or the discovery of the household refrigerator by Fred W. Wolf in 1913 called "Refrigerating apparatus," known at the time as "Domelre" (4) .

Theoretical basis

Most of the cooling processes across all industries (chemical, naval, food, comfort, etc.) are performed at isobaric conditions. The isobaric process operates at constant pressure where the change in pressure is ΔP=0 in the case of heat exchange. Here, when the system heats up and accumulates energy, its volume gets increased and if the system loses heat, its volume contracts. If we refer to the first law of thermodynamics, we can write the equation as follows:

$$Q = \Delta U + W \qquad [1]$$

where W is mechanical work, U is internal energy and Q is heat.

The main limitation of a cooling model in isobar is when the temperature drops below 0°C, and the biological material, with water in its composition, freezes (5).

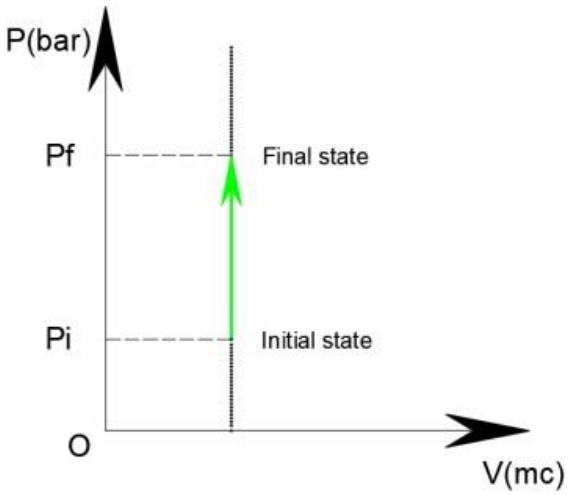

Figure 1. Volume pressure in isochor mode, where V – volume, P - pressure; V = constant, Pi > Pf.

Etymology of word "isochoric"

In the case of an isochoric process, the mechanical work of the system is "0." The only difference in the case of heat transfer can be given by the pressure variation inside. If we trace the process into a pressure-volume chart, we obtain the diagram from Figure 1.

In terms of definition, the word "isochor" means a line representing the variation in pressure, depending on the temperature when the volume of the substance studied is constant (6). It comes from the Greek word "isos" meaning "equal" and "choros" meaning "space." The word is also used with more specific meanings in "isochore" genetics, where it means a large area of DNA with specific properties (7) or in "isochore map" geology, where it means a contour representing dots, specifying the vertical thickness of the layers (8).

History of isochoric preservation

Technical procedures that functioned based on heat transfer in the isochor regime were thoroughly studied following the discovery of the principles of thermodynamics. The research was based a specific phenomenon; the heating of fluid present in a container with a constant volume.

In the first stage of research, cooling was studied with early research focusing on the study on the study of the preservation of different biological materials in isobar mode by the addition of various cryoprotectants in distinct stages of studies (9), or the rapid cooling of liquids until their vitrification, to avoid freezing water inside the studied biological materials (10).

The first notable paper on this topic was published in 1912, which studied the effects of high hydrostatic pressure and water phase diagram. For the first time, this paper also defined the "triple point of water," which is at a temperature of −22 °C and at a pressure of 207.5 MPa. This demonstration opened the door to further studies on the preservation under high pressure conditions, as, at a negative temperature, water can exist in both a liquid and solid state at the same time (11, 12) (Figure 2).

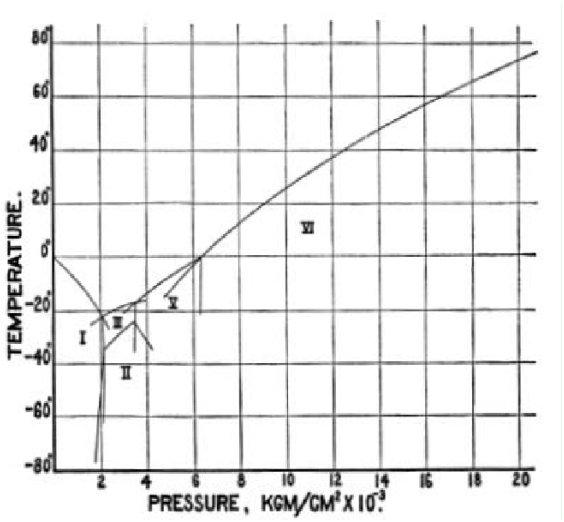

Figure 2. Water phase chart by Bridgman (Original, 1912) (11)

As shown in Figure 2, Bridgman stated that water can exist in the liquid phase and in five types of ice (ICE I, II, III, IV, V). The differences in ice types are given by the differences in conditions necessary for its formation (pressure, temperature).

The discovery that we could preserve numerous prokaryotic and eukaryotic organisms for later use, after cooling them to a temperature of −196 °C, was of huge importance in scientific fields including biology, medicine, and chemistry. However, this would not have been possible without the existence of cryoprotectants.

The best definition of cryoprotectants was published in 1974 by Armand Karow: "A cryoprotectant is any additive that can be added to cells before they are preserved by freezing, thus increasing their survival rate after thawing against the situation in which we would not add anything" (13, 14). Cryoprotectants reduce the risk of destruction of lipids in membranes, proteins, and nucleic acids, while maintaining the integrity of the cell membrane (15).

All cryoprotectants are hydrophilic and can form close bonds with hydrogen in water. This allows them to greatly delay ice formation, even if the necessary temperature and pressure conditions are met (for solvents under study, which may be simple or compound) under normal atmospheric conditions. Mixtures consist of two, or more, basic substances and are obtained because of physical phenomena. Phases disperse between them, but chemical bonds do not break. The chemical properties of the components remain unchanged, but the physical properties of mixture and individual components differ from each other (16).

The concept of cryopreservation emerged when Hans Molisch studied plant freezing with a cryomicroscope using an incipient technology. He concluded that the composition and concentration of substances in plant cytoplasm had a defining role in their survival rate after freezing (17). In addition to this, his discussions with his colleague at the time concluded that the exposure of plants to negative temperatures leads to the accumulation of sugars in them, although this did not mean that sugar could function as an inhibitor of crystallization (18). The importance and recognition of sugars as cryoprotectants was first made in 1912 by Maximov (19, 20).

The successful use of cryoprotective agents began in 1949, when the benefit of using glycerol as a cryoprotectant was first demonstrated. Polge, Smith, and Parkes studied the beneficial properties of cryoprotectants in living biological matter (red cells). Their work was continued and from Lovelock's research, in 1954, results were also published for DMSO, along with other aqueous solutions with low molecular mass such as methanol or acetamide (21). The first synthesis of cryoprotective agents studied in cryobiology for tissue preservation was published in 1969 by AM Karow, Jr. This is known as Karow's “list of 56”. The list contained 56 solutions with cryoprotective properties, according to Table 1.

An interesting aspect is that the 127 studies reported with the 56 solutions many had cryopreserving potentials. Some were in the early stages of studies: glycerol (15 studies), dimethyl sulfoxide [DMSO] (9 studies); glucose (7 studies), sugar (8 studies) and methanol (5 studies).

In 1986, Ashwood-Smith published a paper titled, "Mechanism of cryoprotectant action." Based on studies between 1969 and 1986, there were 20 effective cryoprotectant solutions

remaining (22). Karow's "list of 56," however, remained viable in other branches of science, such as aquaculture. In Nai Hsien-Chao's 1996 paper, 52 cryoprotective solutions were studied for the "Cryopreservation of finfish and shellfish sperms," (23). Among the list of cryoprotective solutions in 1969, many were still included in this later study. High efficacy solutions (relative to the solutions in the table) included dextran, ethylene glycol (relative to fish spermatozoa), hydroxyethyl starch, methanol, polyethylene glycol, polyvinylpyrrolidone and sucrose (23).

Elliot et al. summarized several studies from this field (24). The authors determined that DMSO was the most effective cryoprotectant available at that time. By 2021, in "The need for novel cryoprotectants and cryopreservation protocols: insights into the importance of biophysical investigation and cell permeability," it was concluded that the most commonly used cryoprotectant solutions at this time were DMSO and glycerol (25).

METHODS

Firstly, to study the origins of isochoric preservation, we searched using the WoS Database. A search with the world “isochoric” returned 488 results. A more specific search of the term “isochoric freezing” returned 94 results. From the results of these searches, we then selected the 12 most relevant articles. We arranged the studies chronologically, starting as early as 1912, a year when we found one of the most inspiring manuscripts to include in this review.

RESULTS

The behaviour of various biological materials under these conditions has been studied in detail, especially over the last 50 years.

A study was published in 1977 by Charm, in which the author observed the unusual behaviour of water phase change in isochoric freezing. They studied samples of cod and red crab fillets that were preserved for 36 d at a constant volume at −3°C and 24 MPa. After the retention period, they observed that when they compared the sample kept in isochoric mode with one kept in isobaric mode, the one kept in isochoric mode retained quality for 24 h longer, i.e., the sample remained unaltered for 7 d vs 6 d (26).

Some of the first records of treatment from a mathematical / thermodynamic / chemical viewpoint was published in 1981, when the first paper studying heat transfer at negative temperatures (−40°C) was published. However, the results obtained by theoretical calculations were only estimated and achieved by overlapping theoretical determinations in the 28-120°C temperature range. The temperature-pressure function was theoretically extrapolated to the negative temperatures below 0°C to -40°C. (27). The interesting aspect of this research was that there were differences between the theoretical calculations and practical measurements. This then led to further studies, extending to

Table 1. Effect of preservation on the ΔMass, thickness, soluble solids and moisture content for thawed spinach (33). The values followed by letters in the same column represent homogeneous groups with 95% confidence interval.

Sample	ΔMass %	ΔThickness %	Soluble solids %	Humidity %
Fresh	-	-	8.28 ± 0.05b	91.12 ± 0.51ab
Isochoric preservation – 1 day	41.2 ± 25.9	-6.8 ± 5.6	8.05 ± 0.86b	90.93 ± 0.39a
Isochoric preservation, 7 days	27.2 ± 10.1	-30.7 ± 7.2	7.57 ± 0.91b	90.74 ± 0.41ab
Isobaric preservation – 1 day	3.8 ± 15.5	-30.5 ± 5.1	8.03 ± 0.75b	89.74 ± 1.03abc
Isobaric Preservation – 7 days	-7.9 ± 18.1	-34.4 ± 14.1	11.07 ± 0.09a	86.34 ± 0.69bcd
Packed – Isobar – 1 day	-32.6 ± 11.2	-59.6 ± 4.4	7.13 ± 0.34b	89.04 ± 0.50b
Packed – Isobar – 7 days	-31.7 ± 7.4	-69.9 ± 3.3	8.13 ± 0.83b	86.95 ± 0.17c
Commercial	-	-	4.41 ± 0.03c	85.04 ± 0.52d

temperatures of -180 °C.

In 1982-1984, when Magee and Kobayashi studied isochoric behaviour in various gas mixtures at temperatures between 140 to 273.15 [K], they highlighted the importance of studying the behaviour from the perspective of P-o-T, for both pure solutions and mixtures. Their contributions involving H2-CH4 (28) or 0.2005H2+0.7995CH4 (29) made huge improvements to gas transmission and liquefaction procedures.

With increasing applications of the isochoric process, the behaviour of biological materials received greater attention. In 1988, a mathematical model was defined for simulating cell behaviour at low temperatures. The analysis was based on an irreversible thermodynamic method that demonstrated the destruction of cells when the liquid in which they were immersed freezes. This was shown to be caused by ice that formed internally (30).

A study published in 1990 estimated the frequency of freezing, based on a physico-chemical model using a classical heterogenous freezing theory. With this method, a thermodynamic model operating up to −35°C could be determined (31).

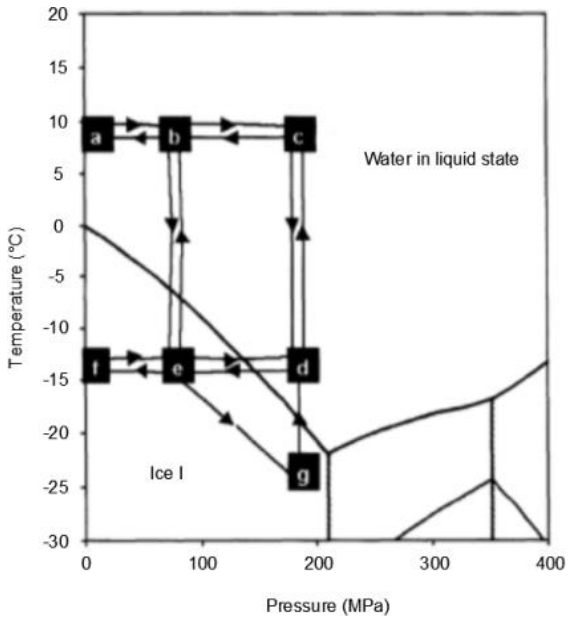

Figure 3. Water phase diagrame. (acdf), Phase change occurs due to a pressure change; (abef), phase change occurs at constant volume; (fdca), phase change (melting) starts by pressure change and continues at a constant volume (21).

In the same year, a separate study in the field of climatology analysed the balance of melting ice under high pressure conditions (0 to 2100 Bar). Based on data gathered from practice, a series of equations were specified to calculate the amount of ice that melts under different conditions. This study further developed specific thermodynamic phenomena using the isochoric regime (32).

It became obvious in 1998 that studying the behaviour of liquids at high pressures in isochoric conditions had become an important area of research when a summary of research done in this field was published by Knorr et al. (33). They defined for the first time the following types of preservation:

- pressure-assisted freezing - phase change occurs at constant volume;
- pressure-shifted freezing - phase change occurs due to pressure change;
- pressure-induced freezing - phase change initiating pressure change and continuing at a constant volume.

In Figure 3, we can observe the transposition of their theory in a water phase diagram.

ISOCHORIC PRESERVATION

In 2005, the results of the afore-mentioned studies culminated in the publication of the following work, "The thermodynamic principles of isochoric preservation," (34). This paper proposed the thermodynamic principles of isochoric cryopreservation. From the explicit mathematical model, we can calculate the amount of ice that is formed in a constant volume system, for water and different water mixtures with other solutions. Theoretical data supported by a series of practical determination to demonstrate the veracity of the methods was presented. Some fundamental principles of the cryopreservation process are also defined in this paper, relating to a two-phase thermodynamic system where water and ice coexist in thermodynamic equilibrium, and temperature and pressure are interdependent. In an isochoric system, the volume is constant in a two-phase isochoric system at a certain pressure and temperature; the only variables that can be adjusted to keep the system in balance is the "quality" of the system and the relative percentage of ice and water in the system. The quality at each temperature or

pressure completely specifies the isochoric cryopreservation process.

In addition, we can add some recent conclusions. We may have a predictable amount of liquid and ice in the isochoric system, depending on the liquid (cryopreserving solution) that we use. As we lower the temperature, theoretically, the number of crystals will increase and it is important to study this with each type of preservation liquid, and optimal temperature at which we will have to cool it so that we have little pressure and decreased ice crystal formation (36).

We can end up in a situation where there is a supercooled liquid without ice crystals in the composition. In terms of microscopic disturbances, some solutions are stable during cooling. In this case, transport protocols can be developed without the formation of crystals and are stable and safe (37).

In the case of mixtures of liquids (water + cryopreserving solutions), we can reach vitrification without the formation of ice crystals during the transformation. We can also reduce the pressure at which this transformation occurs by optimal mixing of component solutions (38).

After defining the principles, several papers were published that analysed different phases of this phenomenon. In 2006, the paper "Analysis of isochoric subcooling" explored the use of isochoric conservation for possible preservation of different organs (39).

In another study published in the same year, "Pressure-assigning freezing and thawing: principles and potential applications," a similar conclusion was reached, namely, the viability of using such a method for the long-term preservation of embryos, organs or cells (40).

Mechanisms of tissue destruction

We can maintain the mechanisms of tissue destruction when preserving in isobar conditions. Blood cannot contain any preserving solutions because when it freezes, it rejects any cryoprotectants that are concentrated around cells, which can result in the following: (i) dehydration of cells, (ii) precipitation of the solution, (iii) changes in pH, and (iv) chemical damage.

There are some limitations of cryopreservation in isobaric conditions, which makes isochoric preservation a viable method. These include the fact that large organs cannot be preserved because the transfer of heat from liquid to organ takes a long time. Cryopreserving solutions themselves could be dangerous for organs that are intended to be preserved. However, these limitations could be avoided using an isochoric preservation technique (41).

Applied studies for isochoric preservations

Since 2016, many studies have been published that have analyzed the behavior of several biological materials in the isochor mode. Here we introduce the findings of 12 key studies.

1) Multicellular organism (*Caenorhabditis elegans*) – This was the first evidence that a living organism may survive isochor preservation without adding cryoprotectants (42) (Fig. 4).

2) Tilapia (*Oreochromis aureus*) fish muscle – A comparison of isochoric and isobaric preservation showed that following isochore preservation, there was no cellular dehydration and morphology of tissues remained intact, as opposed to isobaric preservation (5) (Figs. 5, 6).

3) Potatoes – Potatoes were preserved at −5°C in isochoric conditions, and their tissue colour did not undergo noticeable (enzymatic) change. In contrast, potatoes preserved in isobaric at −5°C had major weight loss, and their colour turned brown. From the microscopic analysis, it became clear that the structural integrity of the potatoes was not altered during isochore preservation, unlike during isobaric preservation (43) (Fig. 7).

4) *Escherichia coli* – After 12 h of isochoric preservation at −15°C and −20°C, the *E. coli* population being studied was reduced by 99.9%. However, in isobaric preservation, it was reduced to 90%. This study showed that high pressure with low temperature, in isochor mode, near the triple point of water, may be more disadvantageous to biological material than the combination of high concentration, low temperature and crystallization, i.e., elements specific to isobaric cooling (44).

5) Rat hearts – After preserving in UW (University of Wisconsin) solution, at −4°C, in isochor mode, hearts had similar properties (Fig. 8A) to those preserved in isobar mode. However, histopathological examination showed that interstitial edema in isochor preserved hearts occupied a much smaller area than those preserved in the isobar mode. The same research was performed at lower temperatures (−6°C; −8°C), and resulted in massive damage to the hearts. Thus, the authors concluded that this type of preservation could be viable up to a temperature of −4°C (Fig. 8B) (45).

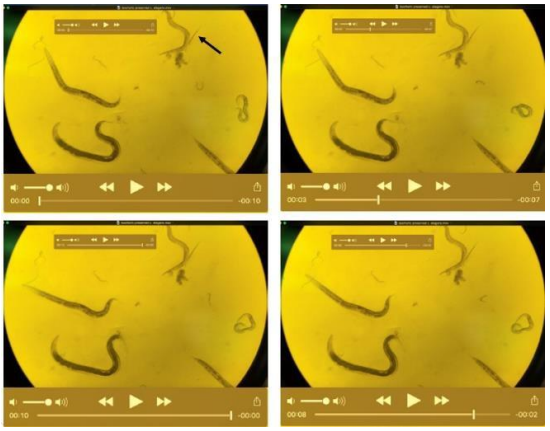

Figure 4. Images illustrating the viability assessment of *Caenorhabditis elegans* (captured from a 10s film). Only the arrow-marked larvae did not move, and the rest of the adult larvae moved (42).

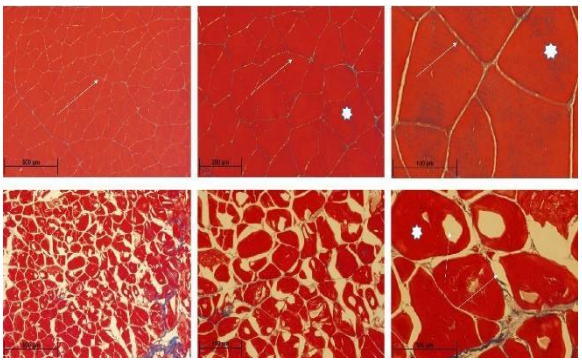

Figure 5. Comparison between fresh muscle tissue (top row) and tissue preserved 3 h to −5°C in isobar regime (bottom row). The fiber is marked with a star and is surrounded by connective tissue. The fibers decreased, and the area of connection between them increased. Their polygon shape was destroyed (5).

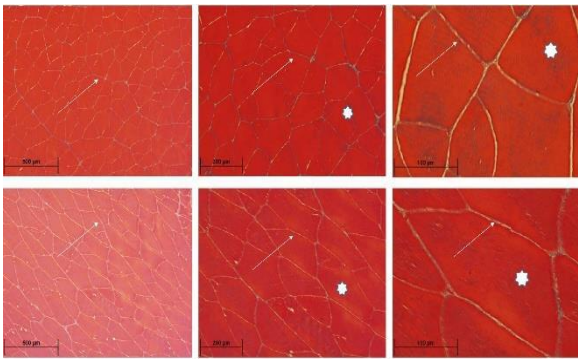

Figure 6. Comparison between fresh muscle tissue (top row) and tissue preserved 3 h to −5 °C in isochor mode (bottom row). The fiber is marked with a star and is surrounded by connective tissue. There are no visible changes to the fibers and tissues resulting from conservation (5).

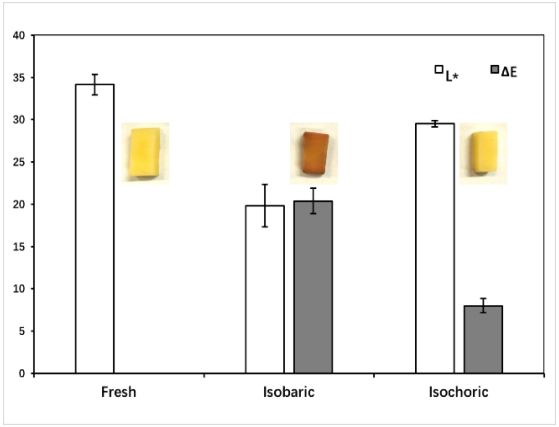

Figure 7. Colorimetric measurements – after potato storage at room temperature, isobaric preservation, and isochoric preservation (30). ΔE (grey bars) and L* (white bars). L* - represents the brightness of the color (L*=0 indicates black and L*=100 indicates diffuse white), a* represents redness and b* represents the yellow in the palette. ΔE is the total colour difference calculated using $\Delta E = \sqrt{\Delta L2 + \Delta a2 + \Delta b2}$, where ΔL, Δa, and Δb is the differences in L*, a*, b*, values before, and after, preservation for each sample (43).

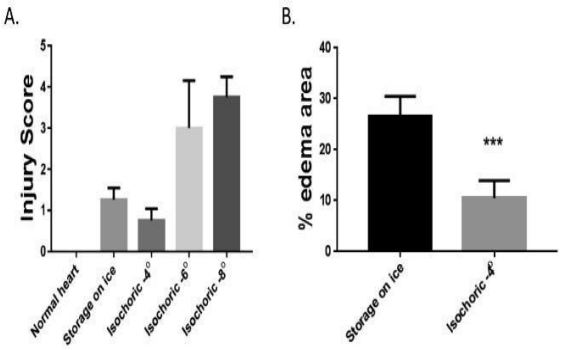

Figure 8. Histopathological assessment of rat hearts. (A) – The result of destruction, considering, as a reference value, the structural integrity of myocytes, the regular arrangement of sarcomas, interstitial edema, the presence of contraction bands and swelling of myocytes. (B) – Percentage area occupied by interstitial edema in hearts preserved on ice, isobar conditions and −4°C (40.62 MPa) in isochoric conditions (45).

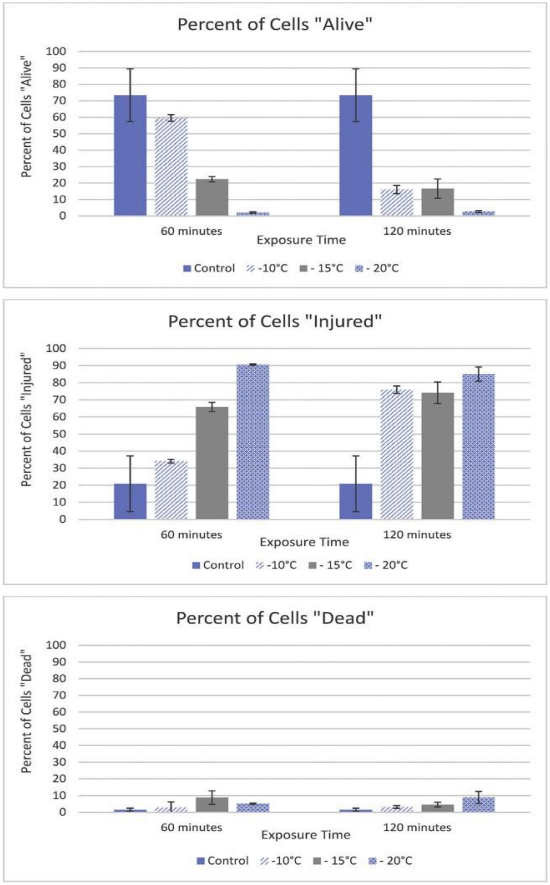

Figure 9. After 60 and 120 min, percentage of cells "alive" (upper), "injured"(middle) and "dead" (lower) after exposure to the following temperatures and pressures: control (37°C, 0.101 MPa), −10 °C (96.5 MPa), −15 °C (162 MPa) and −20 °C (205 MPa) (36).

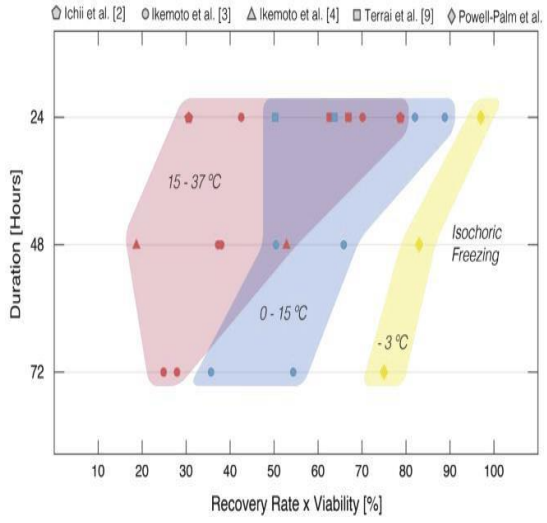

Figure 10. Comparison of the results of the study with previous research within the same time frame relative to cell viability, shown using fluorescent light (47).

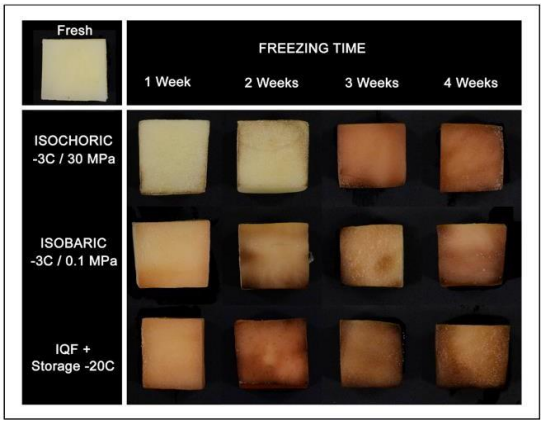

Figure 11. Fresh potatoes versus potatoes thawed after a defined time, preserved in isochor, and isobar, conditions. The samples were thawed for 3 hours at the temperatures shown before the pictures were taken (49).

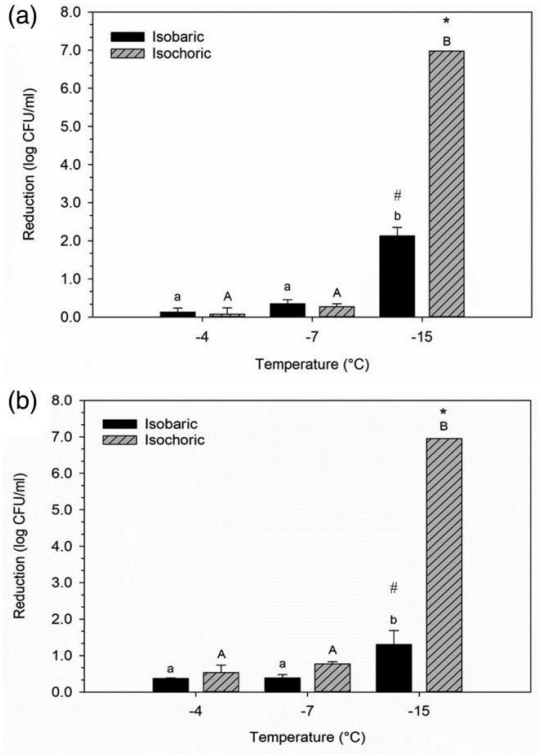

Figure 12. Average reduction in *Listeria monocytogenes* (a) or *Salmonella typhimurium* (b) after isobar or isochor preservation at −15°C for 1, 2, 3, 6, 12 or 24 h (n = 3). Significant differences in the reduction of bacteria were observed after different times in isobar conditions and were marked in lowercase letters (a, b, or c). Significant differences in bacterial reduction were also observed (50).

6) Mammalian cells (MDCK) – The cells were preserved in a phosphate-buffered saline solution in isochoric and studied at −10°C, −15°C, and −20°C, at intervals of 60 and 120 min. By comparison, the number of live/death cells was determined using the cell viability test and flow cytometry. Results of the study showed that about 60% of cells survived at -10°C for 60 min, while 18% survived for 120 min at the same temperature (Fig. 9) (36).

7) Cherries – Cherries preserved at −4°C, in isochor, had the same quality and nutritional properties as fresh ones. Using isochoric preservation, the texture was preserved with minimal weight loss. Simultaneously, their color was the same as that of fresh cherries, while the levels of ascorbic acid, phenolic composition and antioxidant activity was preserved inside them because the preservation was performed without crystallization. Thus, the quality of the fruit preserved was significantly superior to that of the fruit preserved in isobar mode (46).

8) Pancreatic cells – The recovery rate and viability of cells preserved using this method (no cryopreserving osmotic agents used) was found to be significantly higher than using the isobar method (Fig. 10) (47).

9) *Spinacia oleracea* – The properties of spinach preserved at −4°C in both isobar and isochor mode was compared with those of fresh spinach. The spinach preserved in isobar mode exhibited weight loss and damaged texture after thawing and less nutritional value due to isobar preservation, whereas spinach kept in isochor mode retained its properties and its nutritional value was found to be increased due to preservation. Table 1 shows the properties of spinach under different conditions (48).

10) Minimally processed potatoes (*Solanum tuberosum*) – The isochoric-preserved samples (−3 °C / 30 MPa) were compared with isobar-preserved samples (−3°C/0.1 MPa) and rapid cooling at −20°C followed by storage for 4 weeks. Different properties such as microstructure, texture, colour nutritional value (ascorbic acid, phenolic content, and antioxidant capacity) and the activity of polyphenol oxide was compared. Samples kept in isochor showed improvements in all the above- mentioned variables. All methods caused brownish staining of the samples in the case of isochore preservation; however, only following more than one week (Fig. 11) (49).

11) Onocytogenes of *Listeria* and *Salmonella typhimurium* – The survival rate of bacteria following isochoric and isobaric preservation was compared. They were also examined structurally after preservation using microscopes. The study showed that isochoric preservation at – 15 °C for 24 h reduced the bacteria population to a level that made them stranded. Furthermore, the study showed that isochoric cooling can successfully contribute to the reduction of pathogens (Fig. 12) (50).

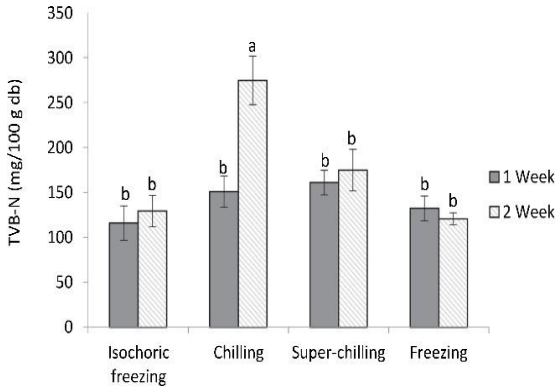

Figure 13. Effect of preservation on the content of TVB-N in tilapia fillets (51).

12) Tilapia fish filets (*Oreochromis aureus*) – Isochoric preservation has been compared to cooling, super-cooling and freezing. Isochoric preservation showed colorimetric degradation like cooling and showed a similar texture of preserved sample compared to the fresh one. TBARS for isochor-preserved samples were like those in fresh fish, unlike samples preserved by other methods where it was seen to be increased by 53%, 55% and 34%. The volatile nitrogen (TVB-N) content was 1.4 times higher for isochor-preserved samples as opposed to other preservation methods, where it was higher by 3.0, 1.9 and 1.3, respectively (Fig. 13). In the microstructural study, no cellular damage was reported following isochoric preservation (51).

DISCUSSION AND CONCLUSIONS

To date, all the published research has led to the recognition of isochoric preservation as a viable method used in the field of food technology and nutrition, among other pioneering methods in the field. The model is well recognized, so further research in years to come will continue to develop and streamline the mechanisms involved so that they can be used on a much wider scale

(52).

The general conclusion is that isochoric preservation, without the possibility of the presence of ice within the cells under study, is still considered a promising technique in order to extend the shelf life of various biological materials compared to traditional methods of preservation. In this context, we have shown in our review that many studies have analyzed isochoric preservation methods and cryoprotectants, but they have mostly been studied individually.

Reference is made to alternative modes of preservation (such as in isochor) in articles involving cryoprotectants (53) and vice versa (54). The value of cryopreservation has been achieved by proving its effectiveness as a viable method in areas such as food safety and nutrition (49, 50) and medicine (55). Simultaneously, the method has huge potential from an economic perspective (56). By combining the "isochoric systems" and the "cryopreserving substances", we hope to try to diversify our portfolio of cryoprotective agents (25) and develop specific solutions for this cooling method.

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