Isochoric Supercooling of Human Induced Pluripotent Stem Cell(hiPSCs) Derived Cardiomyocytes in Cardiac Microphysiological Systems (MPS)

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Abstract
Organ preservation over extended periods of time presents a significant challenge in the field of organ transplantation due to the development of ischemia damage, which can lead to organ failure. We explore a novel form of preservation, isochoric supercooling, to preserve hi-PSC derived-cardiomyocytes in cardiac MPS. We investigate this via viability and structural analysis. Results show significantly high viability in revived cardiac microtissue. The findings hold significant implications for cardiac research and cryopreservation fields, offering new advancements for drug screening, disease modeling, and tissue engineering. Furthermore, the research emphasizes the importance of carefully optimizing experimental conditions for successful isochoric cryopreservation.

Introduction
The heart organ relies on healthy cardiac tissue made up of a variety of cells, such as cardiac progenitor cells or endothelial cells. For our research, we focus specifically on cardiomyocytes due to their ability to contract and their high prevalence in myocardial tissue. However, preserving cardiac microtissue for in vitro, or regenerative medicine purposes presents significant challenges due to their sensitivity to temperature changes and potential damage during cryopreservation processes.

Traditional cryopreservation techniques involve the use of cryoprotectants and slow cooling to prevent ice crystal formation, which can cause cell damage and alter the cellular structure. Despite these efforts, the process can lead to cellular volume changes during freezing and thawing, negatively impacting cell viability and function. Isochoric supercooling, a relatively novel approach, offers a potential solution to these issues.

Isochoric supercooling is a cryopreservation method that aims to freeze biological materials, such as cells, without altering their volume, and it relies on rapid cooling to avoid ice crystal formation. This unique approach allows the preservation of cellular structures and functions with minimal damage, potentially improving cell viability after thawing.

Previous studies have explored the use of isochoric supercooling in preserving various cell types, such as red blood cells (Beşchea, George-Andrei, et al, 2012). These studies have shown promising results in terms of post-thaw viability and functionality. However, its application to cardiac microtissue remains relatively unexplored.

The main purpose of this experiment is to investigate the feasibility and effectiveness of isochoric cryopreservation for cardiac tissue. By employing advanced techniques and equipment, we aim to assess...
the viability, structural integrity, and contractile function of cardiomyocytes after undergoing isochoric supercooling and subsequent thawing. The successful application of isochoric supercooling to cardiomyocytes could offer significant advantages over traditional cryopreservation methods, potentially leading to improved storage and transportation for research and clinical applications.

Methods

**Chip Fabrication** - The cardiac chips were fabricated using a two-step process. First, Polydimethylsiloxane (PDMS) was prepared by mixing a PDMS base and curing agent in a 10:1 ratio. The mixture was degassed to remove air bubbles and then poured onto a silicon wafer mold with microfluidic channels. After curing at 70°C for 2 hours, the solidified PDMS was carefully peeled off to obtain the cardiac chip structure.

**Oxygen Plasma Bonding** - To ensure a sealed microfluidic structure, the PDMS chips and glass cover slides were exposed to oxygen plasma bonding. Both surfaces were treated with oxygen plasma to create reactive oxygen species, allowing for a covalent bond formation when pressed together. The bonding process was carried out under controlled conditions to ensure the proper alignment of microfluidic channels.

**Cell Loading** - Mature cardiomyocytes were differentiated from hiPSCs (human-induced pluripotent stem cells. The cells were then suspended in a culture medium, and the media was carefully introduced into the microfluidic channels of the cardiac chips. The chips were placed in an incubator for a specified period to allow cell attachment and stabilization.

**Isochoric Cryopreservation** - The isochoric cryopreservation was conducted using a custom-designed isochoric pressure chamber. The chamber was equipped with precise temperature and pressure control mechanisms. The process involved lowering the temperature inside the chamber to -3°C, while maintaining the pressure at the original level. This rapid cooling procedure avoided the formation of ice crystals and prevented changes in cell volume, thus preserving the integrity of the cardiomyocytes.

The cardiac chips, loaded with cardiomyocytes, were carefully placed inside the isochoric pressure chamber. The chamber was then sealed to create a controlled environment for cryopreservation. The isochoric cryopreservation process was carried out for 24 hours, allowing the cardiomyocytes to reach the desired low temperature without any pressure-induced volume alterations.

**Data Evaluation** - When observing and imaging the chips, we looked for three main categories:

1. Survival: Did the cells survive after the isocirc supercooling?
2. Mechanical Function: Is the tissue contracting as a whole or partially? Are there any potential irregularities/arrythmias?
3. Electrical Function: What is the EKG (in beats per minute)? Are the cells contracting and pumping calcium?
Results
The results of the isochoric cryopreservation experiment demonstrated promising outcomes regarding the preservation of cardiac microtissue viability within the chips. Quantitative analysis of microscope images revealed that a significant proportion of the cryopreserved tissue exhibited preserved viability.

For Chip 2c shown in Figure 1, the GFP image before preservation and 1 hour post preservation both show large amounts of calcium pumping through the tissue in an evenly distributed manner. However, the chip died 24 hours post preservation, as shown by the lack of calcium (green fluorescence).

![Figure 1: GFP and regular images of Chip 2c](image)

On Chip 3b in the same experiment (images shown in Figure 2), there was a similar trend of revival after cryopreservation. Before preservation and 1 hour post-preservation does have some calcium flux. However, calcium quenching is also present. Calcium quenching is when some cells in the microtissue take up more calcium than needed and therefore deprive other cells from the calcium needed for contractility. This can lead to cell apoptosis, as shown in the 24 hours post preservation. What’s interesting in this chip is that there is significant cell revival 48 hours post-cryopreservation. We can see that the tissue is fuller and contracting strongly, and there is fluorescence in the GFP image. The results of Chip 3b signifies successful preservation of the tissue over 2 days after isochoric supercooling.

![Figure 2: GFP and regular images of Chip 3b](image)
Discussion

Delicacy of Cardiomyocytes
The data obtained from this study has considerable significance in various areas of cardiac research and regenerative medicine. It also showed the delicate nature of cardiomyocytes and their sensitivity to environmental factors during cryopreservation. It was observed that while isochoric cryopreservation showed promise in preserving cardiomyocyte viability and structural integrity, the success of the process was influenced by factors such as the cell culture environment and handling procedures.

Subtle variations in the culture conditions and cell preparation techniques appeared to impact the viability and functionality of the cryopreserved cardiac microtissue. Factors such as the quality of the culture medium, the handling of cells during suspension loading, and the stability of microfluidic channels all played a critical role in determining the success of the cryopreservation process.

Overall, isochoric cryopreservation's ability to preserve viable and structurally intact cardiomyocytes in cardiac microtissue offers new avenues for advancing cardiac research and drug development.

Drug Screening and Cardiomyocytes
Preserving functional microtissue within cardiac chips allows for more realistic and accurate drug screening assays. The cryopreserved cells can be utilized to test the effects of various pharmaceutical compounds on cardiac function, enabling the identification of potential therapeutic candidates for cardiac diseases. Additionally, the preserved cardiomyocytes can serve as an invaluable resource for studying the molecular mechanisms underlying cardiac pathologies and physiological responses.

Regenerative Medicine and Tissue Engineering
The preservation of viable cardiomyocytes within cardiac chips has promising implications for regenerative medicine and tissue engineering. Cryopreserved cardiomyocytes can be used to engineer cardiac tissue constructs for transplantation, providing a potential therapeutic option for patients with heart injuries or diseases. Furthermore, the ability to create biobanks of cryopreserved cardiomyocytes enhances the accessibility and availability of functional cells for various research and clinical applications.

Conclusion
This research demonstrates the efficacy of isochoric cryopreservation for maintaining the viability and structural integrity of cardiomyocytes within cardiac chips. The data obtained from this study emphasize the potential of isochoric supercooling as an innovative cryopreservation technique with significant implications for cardiac research, drug screening, and regenerative medicine. The preservation of functional cardiomyocytes within cardiac chips provides a valuable resource for advancing our understanding of cardiac physiology and pathology and holds promise for future clinical applications in the field of cardiovascular medicine.

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