

Polyethylene glycol and caspase inhibitor emricasan alleviate cold injury in primary rat hepatocytes

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ABSTRACT

Current methods of storing explanted donor livers at 4 °C in University of Wisconsin (UW) solution result in loss of graft function and ultimately lead to less-than-ideal outcomes post transplantation. Our lab has previously shown that supplementing UW solution with 35-kilodalton polyethylene glycol (PEG) has membrane stabilizing effects for cold stored primary rat hepatocytes in suspension. Expanding on past studies, we here investigate if PEG has the same beneficial effects in an adherent primary rat hepatocyte cold storage model. In addition, we investigated the extent of cold-induced apoptosis through treating cold-stored hepatocytes with pan caspase inhibitor emricasan. In parallel to storage at the current cold storage standard of 4 °C, we investigated the effects of lowering the storage temperature to −4 °C, at which the storage solution remains ice-free due to the supercooling phenomenon. We show the addition of 5 % PEG to the storage medium significantly reduced the release of lactate dehydrogenase (LDH) in plated rat hepatocytes and a combinatorial treatment with emricasan maintains hepatocyte viability and morphology following recovery from cold storage. These results show that cold-stored hepatocytes undergo multiple mechanisms of cold-induced injury and that PEG and emricasan treatment in combination with supercooling may improve cell and organ preservation.

1. Introduction

Liver transplantation is a life-saving treatment for end-stage liver disease and liver failure, but the availability of donor organs is limited by factors such as inadequate preservation for extended durations following donor explant [1,2,3]. The University of Wisconsin (UW) preservation solution was introduced in the mid-1980s [4,5,6], and hypothermic liver preservation by immersion in UW solution on ice or at 4 °C, termed static cold storage (SCS), remains widely used today in clinical applications [7,8]. Nonetheless, prolonged storage is well known to increase the risk of graft dysfunction that contributes to complications post-transplantation [9,8]. Encouragingly, groups have made considerable progress developing improved preservation protocols to minimize preservation-related injury and prolong storage duration in *ex vivo* whole livers [10,11,12,13,14,15]. However, although these studies have increased our knowledge of how specific preservation protocols can lead to greater liver graft outcomes, whole organ studies provide limited options for deeper investigations into mechanisms of cellular survival or damage.

On the cellular level, it has been seen that hepatocytes following SCS display characteristics associated with necrosis and apoptosis, with additional detrimental effects that are not yet well understood [16,17,18,19]. Groups have also shown that plated primary hepatocytes exhibit numerous additional molecular responses to hypothermia, including reactive oxygen species generation, mitochondrial stress, and nucleic acid fragmentation [16,20,21,22,23,24–26,27]. Our lab has previously shown that lipid peroxidation contributes to membrane damage in cold stored suspended hepatocytes, and that UW supplemented with PEG can reduce the degree of lipid peroxidation and stabilize the cellular membrane; the same study also showed the beneficial effects of long-term (>3 days) storage at a subzero, non-freezing temperature of −4 °C, at which the storage solution remains unfrozen due to the supercooling phenomenon [24]. Though, suspended hepatocytes are commonly seen to have decreased attachment efficiency post-preservation, offering limited options to assess cell survivability, function, and morphology over extended culture periods post-storage [24,28]. In addition, studies have only assessed suspension hepatocytes for viability and function following storage at subzero, nonfreezing temperatures, which may

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produce different results than plated hepatocytes [29,24,30,28].

Our lab has previously developed a collagen sandwich culture system to mimic hepatocytes' *in vivo* environment and allow for extended culture (>7 days) of primary rat hepatocytes [31]. Using a modified version of the collagen sandwich culture system, we evaluated in this study the viability and metabolic function of cultured rat hepatocytes following hypothermic preservation in UW solution at supercooling temperature -4°C , and compared the results obtained with conventional SCS at 4°C . Expanding on prior work, this study aims to investigate if PEG supplementation in UW solution for cold storage at $\pm 4^{\circ}\text{C}$ will also provide membrane stabilization for adherent hepatocytes similar to previously explored suspended hepatocytes. In addition, we hypothesize that pan-caspase inhibitor emricasan treatment during recovery can improve cold-stored hepatocyte viability. The hepatocytes were stored for 24 h, which is the maximum time that rat and human livers have been stored with UW before being deemed unviable for transplantation [5,10,32].

2. Materials and methods

2.1. Hepatocyte isolation

Primary rat hepatocytes were sourced from the Cell Resource Core at Massachusetts General Hospital (MGH) and were used for all experiments. The animals were maintained per National Research Council guidelines, and the isolation was performed in accordance with protocol #2011N000111, as approved by the Institutional Animal Care and Use Committee (IACUC) at Massachusetts General Hospital (MGH). Hepatocytes were isolated from two to three-months-old female Lewis rats (Charles River, USA), as described previously [10]. Experiments were conducted with cells from isolations resulting in a minimum cell viability of 90 %, as determined by trypan blue exclusion or acridine orange and propidium iodide (AOPI) staining with the Cellometer K2 (Nexcelom Bioscience, USA). Following isolation, the cells were stored on ice for a maximum of 3 h in seeding media. The seeding media consists of Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA), supplemented with 200 units/mL penicillin and 200 $\mu\text{g/mL}$ streptomycin (Sigma-Aldrich, USA), and 10 % fetal bovine serum (FBS) (Gibco, USA).

2.2. Hepatocyte culture

Plastic 24-well tissue-culture plates were first coated with type I collagen (R&D Systems, USA) at $15\text{ }\mu\text{g/cm}^2$. Then, each well was seeded with hepatocytes at a density of 1.25×10^5 cells/ cm^2 in seeding media. Following 1 h of incubation at standard culture conditions (37°C , 5 % CO_2) to allow for cell attachment, the media was changed to culture media. The culture media consists of Williams' E Medium (Sigma-Aldrich, USA), supplemented with 20 ng/mL epidermal growth factor (EGF) (Sigma-Aldrich, USA), 7.5 $\mu\text{g/mL}$ hydrocortisone (Pfizer Inc., USA), 7 $\mu\text{g/mL}$ glucagon (Boehringer Ingelheim, USA), 0.5 unit/mL insulin (Eli Lilly, USA), 200 units/mL penicillin and 200 $\mu\text{g/mL}$ streptomycin (Sigma-Aldrich, USA), and 10 % FBS (Gibco, USA). In culture media, the cells are stabilized overnight at standard culture conditions. The following day, the media was aspirated, and a top gel was prepared in each well by adding 100 μL of type I collagen diluted in sodium bicarbonate-buffered High-Glucose DMEM (Gibco, USA) at a final collagen concentration of 1.25 mg/mL and final pH of 7.4. This solution was incubated at 37°C for 1 h to allow for collagen polymerization to complete the top-gel culture setup. Following polymerization, 400 μL of culture media was added to each well and the cells are stabilized overnight for an additional time at standard culture conditions.

2.3. Cold incubation and recovery

Hepatocytes were stored at two temperatures for 24 h: 4°C to replicate current static cold storage methods (SCS) or non-freezing

subzero -4°C (SZ). The SCS groups were stored in a 4°C fridge while the SZ groups were stored in a portable freezer (MHD13F-DM, Engel-USA Inc., USA). The temperatures were monitored continuously for the fridge and every 4 h for the freezer via thermocouples and were observed to maintain an average temperature of $4.53 (\pm 0.66)^{\circ}\text{C}$ and $-4.18 (\pm 0.23)^{\circ}\text{C}$, respectively. At both storage temperatures, the cells were stored in UW solution without glutathione (Bridge to Life, USA) supplemented with or without 5 % 35 k-PEG (Sigma-Aldrich, USA), resulting in a total of four storage groups. Following storage, the storage solution was collected for later analysis and the four storage groups were each washed with culture media at 4°C . Then, the cold culture media was replaced with 37°C culture media supplemented with or without 5 μM pan-caspase inhibitor emricasan (Selleck Chemicals, USA). The emricasan stock solution is suspended in dimethyl sulfoxide at a concentration of 5 mM, stored at -80°C , and added into culture media at a 1:10,000 dilution immediately prior to cell treatment. The cells were then incubated at standard culture conditions with assessments occurring at 2 and 24 h after recovery from storage with separate plates being used for each assessment. In addition, a group was only maintained at standard culture without cold storage and assessed on the same day as the cold storage groups. The experimental flow is depicted in Fig. 1.

2.4. Morphology assessment

Cell staining was performed using a Live/Dead viability kit, consisting of calcein AM and ethidium homodimer stains, and a Hoescht nuclear stain (all from Thermo Fisher Scientific, USA) as per the manufacturers' protocols. Images were taken with the EVOS M5000 inverted microscope (Thermo Fisher Scientific, USA).

2.5. Cell metabolism assay

Resazurin reduction activity was measured by the PrestoBlue assay (Invitrogen, USA). PrestoBlue reagent was added to 37°C culture media at a 1:9 ratio to create a working reagent and 500 μL was added to each well. The cells were then incubated at standard culture conditions for 30 min. After 30 min, the plate was gently tapped 10 times on the sides to mix, and 300 μL from each well was divided into three wells of a black, flat 96-well plate. The fluorescence was read from the top of the well using the Synergy 2 microplate reader (Biotek, USA) per the manufacturer's protocol to determine cellular resazurin reduction activity. The plain working reagent was measured to determine the background baseline. Resazurin reduction activity are presented as a percentage of the activity in the SCS no treatment group.

2.6. Lactate dehydrogenase release assay

Membrane integrity was assessed by measuring the amount of cytoplasmic lactate dehydrogenase (LDH) released into the medium, using an LDH detection kit (Sigma-Aldrich, USA) following the manufacturer's protocol. The absorbance was read from the top of the well using the SpectraMax iD3 microplate reader (Molecular Devices, USA). LDH activity levels are presented as a percentage of the activity in the SCS no treatment group.

2.7. Caspase 3/7 activity assay

The caspase 3/7-glo (Promega, USA) assay reagent was prepared beforehand by a 1:1 combination with culture media to create a working reagent and 400 μL was added to each well. The plate was then left on a shaker set at 150 rpm for 30 min at room temperature while protected from light. Following 30 min, 200 μL from each well was transferred to a white, flat bottom 96-well plate. The luminescence was read from the top of the well using the Synergy 2 microplate reader (Biotek, USA) per the manufacturer's protocol. The plain working reagent was measured to determine the background baseline. Caspase 3/7 activity levels are

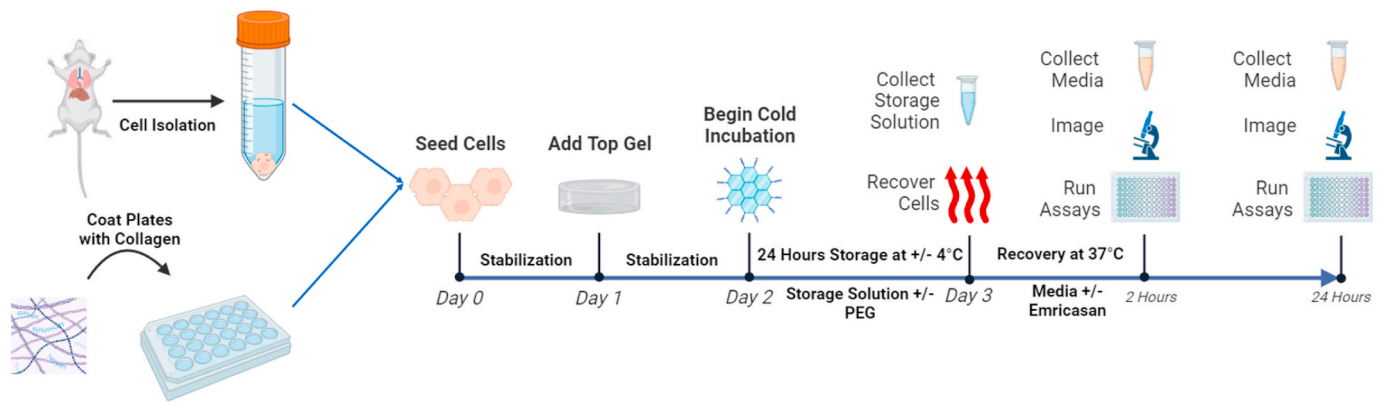


Fig. 1. Experimental Design. Schematic illustration of the experimental design and groups. Made with Biorender.

presented as a percentage of the activity in the SCS no treatment group.

2.8. Reactive oxygen species formation assay

Reactive oxygen species (ROS) formation was assessed by the CellROX Green Reagent (Invitrogen, USA). CellROX Green reagent was added to culture media to create a working reagent of 10 μ M and 500 μ L was added to each well. The cells were then incubated at standard culture conditions for 30 min. Following 30 min, the cells were washed two times with 500 μ L of phosphate-buffered saline and then 500 μ L of 10 % formaldehyde was added to each well. On the same day, images were taken with the EVOS M5000 inverted microscope (Thermo Fisher Scientific, USA). The green fluorescence intensity was determined through image analysis software ImageJ (National Institutes of Health, USA). The fluorescence intensity recorded is correlated with ROS levels and presented as a percentage of the levels in the standard culture group.

2.9. Statistical analysis

Differences in cellular viability markers (LDH, Caspase, Resazurin Reduction) between different temperature and treatment groups were analyzed through a full-effect, two-way analysis of variance (ANOVA). Tukey's post-hoc test was used to compare the mean of every group with each other within their timepoints (0, 2, and 24 h following recovery). Statistical significance is determined by $p \leq 0.05$. All analyses were performed in Prism 10 (GraphPad Software Inc., USA). All error bars in all graphs in the figures denote standard deviation.

3. Results

Cold storage for 24 h at $\pm 4^\circ\text{C}$ results in decreased metabolism, membrane damage, and increased caspase activity in hepatocytes.

We tested the effect of 24 h cold storage on plated rat hepatocytes and observed no change in metabolism between groups at 2 h following recovery from cold storage (Fig. 2a). Following 24 h of recovery, we observed significantly lower cell metabolism in both temperature groups as compared to standard culture, with the SZ storage group showing significantly lower metabolism than the SCS group (Fig. 2a). We also observed greater extracellular LDH activity (Fig. 2b) at 2 h and 24 h and greater caspase 3/7 activity (Fig. 2c) at 2 h following recovery from both storage temperatures as compared to the standard culture group. Additionally, we observed greater caspase 3/7 activity at 24 h in the SZ group when compared to standard culture (Fig. 2c).

3.1. PEG supplementation during storage alleviates membrane damage and increases cell metabolism

We investigated the membrane stabilization effect of PEG during cold storage by supplementing the storage solution with 5 % PEG. The general metabolism of cells measured by the PrestoBlue resazurin reduction activity assay following 2 h of recovery found the SZ-PEG group to have significantly higher metabolic activity than the non-treated cold stored groups and the standard culture group (Fig. 3a). Following 24 h of recovery, all cold storage groups were found to have significantly lower metabolic activity than standard culture (Fig. 3a). Of the cold-stored groups, we observed the PEG-treated groups to have significantly higher metabolic activity than their non-treated counterparts (Fig. 3a).

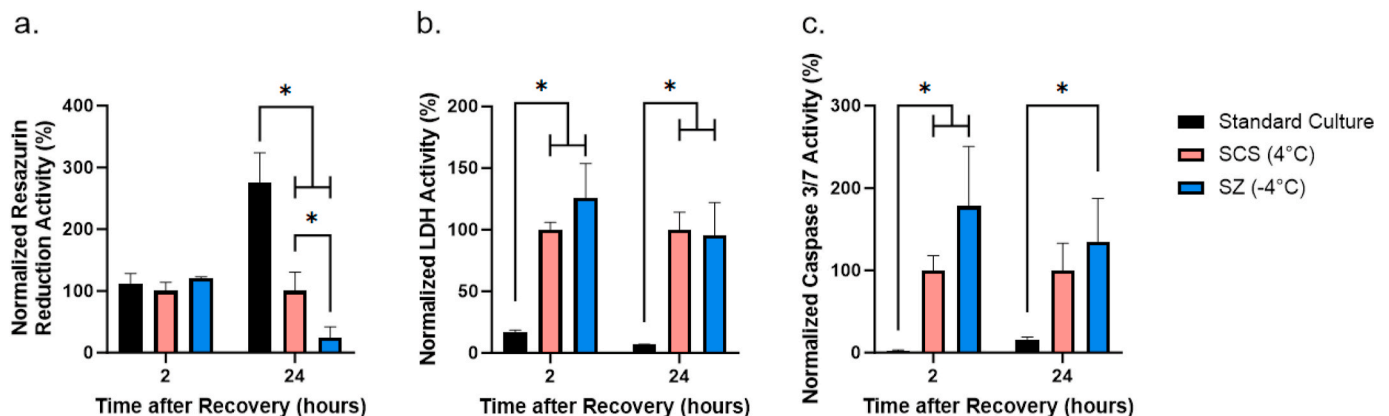


Fig. 2. Effect of non-freezing hepatocyte cold storage at $\pm 4^\circ\text{C}$ on resazurin reduction, LDH, and caspase 3/7 activity. Resazurin reduction (a), LDH (b), and Caspase 3/7 (c) activity is normalized to the activity of the SCS group. * = $p \leq 0.05$ ($n = 3$ biological replicates). Error bars denote standard deviation.

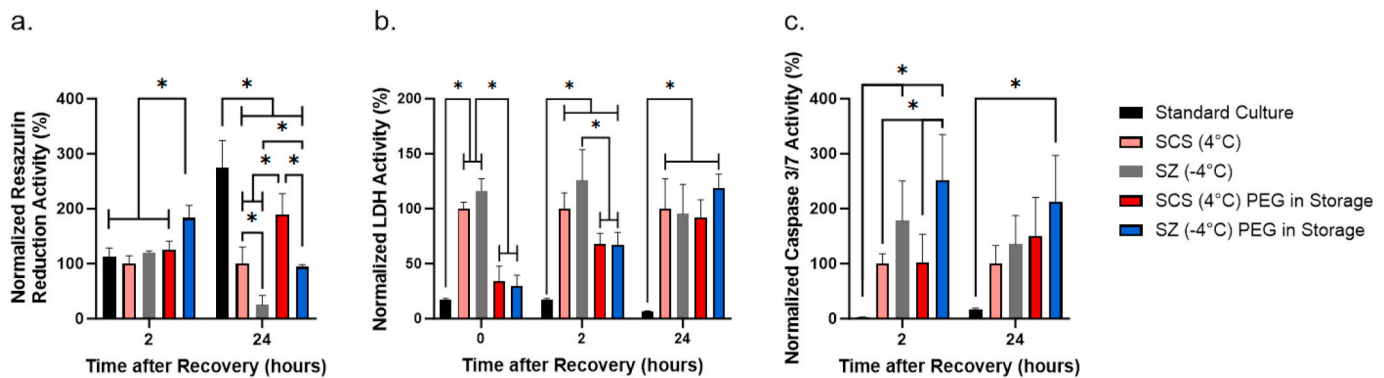


Fig. 3. Effect of PEG supplementation during storage on resazurin reduction, LDH, and caspase 3/7 activity. Resazurin reduction (a), LDH (b), and Caspase 3/7 (c) activity is normalized to the activity of the SCS no treatment group. * = $p \leq 0.05$ ($n = 3$ biological replicates). Error bars denote standard deviation.

The assessed extracellular LDH activity in both SCS-PEG and SZ-PEG groups are comparable to standard culture immediately after storage, and significantly lower than the activity of their non-treated counterparts until 2 h of recovery (Fig. 3b). Following 2 h of recovery, the standard culture group has significantly lower extracellular LDH activity than all the cold storage groups, and of the cold storage groups, we observed the SZ group to have significantly higher activity than both the SCS- and SZ-PEG groups (Fig. 3b). Following 24 h of recovery, no difference was seen in extracellular LDH activity between the cold storage groups (Fig. 3b).

For caspase 3/7 activity following 2 h of recovery, we observed the activity of the standard culture to be significantly lower than the SZ and SZ-PEG groups (Fig. 3c). In addition, we observed the caspase 3/7 activity of the SZ-PEG group following 2 h of recovery to be significantly higher than both the SCS and SCS-PEG group (Fig. 3c). After 24 h of recovery, caspase 3/7 activity of the SZ-PEG group was the only group found to be significantly higher than standard culture (Fig. 3c).

3.2. Emricasan supplementation during recovery alleviates caspase 3/7 activity following cold storage

To alleviate the observed increase in caspase 3/7 activity following cold storage as compared to standard culture (Fig. 2c), hepatocytes were recovered with culture media supplemented with 10 μ M emricasan, a pan-caspase inhibitor. We observed no difference in metabolic activity between all groups following 2 h of recovery, except for the SZ-emricasan group having lower activity than its non-treated counterpart (Fig. 4a). Following 24 h of recovery, all cold storage groups were seen to have significantly lower metabolic activity than standard culture, and of the cold stored groups, SZ and SCS emricasan treatment did not result in significantly greater activity than their non-treated

counterparts (Fig. 4a). However, we observed the SCS-emricasan group to have significantly greater activity than the SZ-emricasan group (Fig. 4a).

In addition, we observed that emricasan has no effect on reducing LDH release in treated groups, with all cold storage groups having greater extracellular LDH activity than standard culture following both 2 and 24 h of recovery (Fig. 4b). However, the addition of emricasan in recovery media significantly decreased caspase 3/7 activity in both SZ-emricasan and SCS-emricasan groups following 2 and 24 h of recovery to levels comparable to standard culture (Fig. 4c). Also, we observed that the addition of only DMSO, the vehicle control, caused no change in either metabolic or caspase 3/7 activity when compared to the respective SCS and SZ groups (Supplemental Figs. 1a and 1b). Furthermore, we have observed no difference in caspase 3/7 activity between emricasan treatment in storage solution versus in recovery media only (Supplemental Figs. 2c and 2d).

3.3. Combined treatment of cells with PEG supplementation during storage and emricasan in recovery results in the greatest cell metabolism

Following treatment with PEG and emricasan individually, we wanted to observe the alleviating potential of a combined treatment on preserving metabolic activity by limiting LDH release through PEG treatment along with inhibiting caspase activity through emricasan treatment (Supplemental Figs. 3a and 3b). Following treatment with PEG in storage and emricasan in recovery, we measured the metabolic activity of hepatocytes at 2 and 24 hours of recovery for the groups that received no treatment, PEG in UW, emricasan in recovery, and combined treatment. At 2 h following recovery from storage (Fig. 5a), we did not observe differences between the no treatment groups and corresponding combined treatment groups at both storage temperatures. At

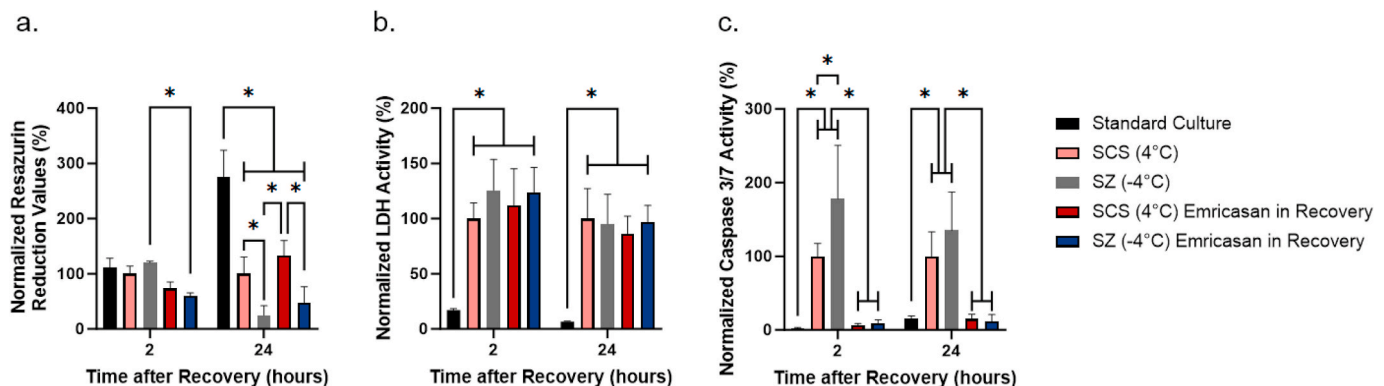


Fig. 4. Effect of emricasan treatment during recovery on resazurin reduction, LDH, and caspase 3/7 activity. Resazurin reduction (a), LDH (b), and Caspase 3/7 (c) activity is normalized to the activity of the SCS no treatment group. * = $p \leq 0.05$ ($n = 3$ biological replicates). Error bars denote standard deviation.

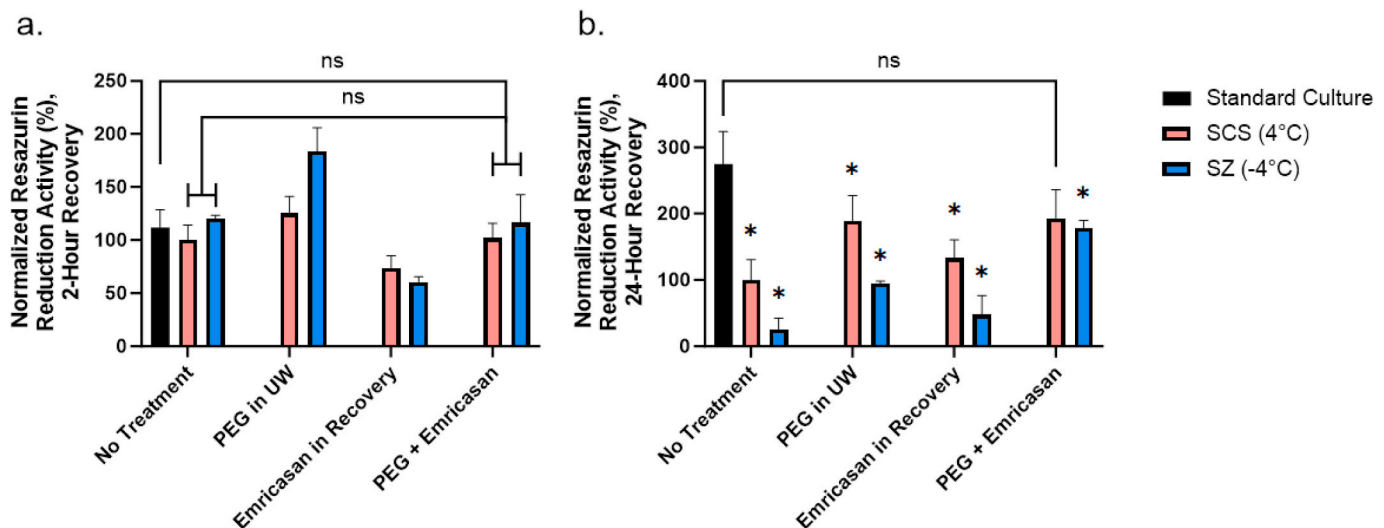


Fig. 5. Combined treatment of PEG and emricasan provides the greatest recovery in general cell metabolism following cold storage. The resazurin reduction activity was determined using PrestoBlue following (a) 2 h and (b) 24 h of recovery. The activity is normalized to the activity of the SCS no treatment group. * = significantly different than standard culture ($n = 3$ biological replicates). Error bars denote standard deviation.

24 h post-storage, we observed that the metabolic activities of the combined treatment groups are significantly higher than corresponding no treatment groups (Fig. 5b), with the metabolic activity of the SCS-combined treatment group being comparable to standard culture ($p = 0.52$). However, we observed that combined treatment did not significantly influence ROS generation following cold storage as compared to no treatments or individual treatments of PEG or emricasan (Supplemental Fig. 4).

3.4. Hepatocyte morphology is preserved following cold storage when the process is supplemented with PEG and emricasan

Phase-contrast and fluorescent microscopy images of hepatocytes were taken following 2 and 24 h of recovery from cold storage (Fig. 6). Without cold storage, standard hepatocyte cultures displayed an expected polygonal morphology (Fig. 6a & b far right images). At 2 h of recovery following cold storage, significant dysmorphology is noticeable in the SZ group, and to less of an extent in SCS (Fig. 6a). Following 24 h of recovery, all the non- and partially treated groups for both storage temperatures displayed varying intensities of nuclear collapse and vacuolization (Fig. 6b). Using the 24 h SCS no treatment group as an example, although a fraction of dead hepatocytes retains their general shape, their cell membrane is permeable to ethidium homodimer (Fig. 6c). In addition, a fraction of dead hepatocytes was not stained by either ethidium homodimer or Hoechst, suggesting a collapse of the nuclear envelope (Fig. 6c). However, the groups treated with PEG and/or emricasan retained healthy morphology to an extent, with the combined treatment group displaying morphology comparable to the standard culture control (Fig. 6).

4. Discussion

Since its conception in the 1980s by Folkert Belzer and James Southard, UW solution for cold storage has increased the quality and duration of preservation for various organs, tissues, and cells [5,8]. With UW solution, whole livers remain transplant viable after up to 24 h of cold storage [5,10,32]. Though, extension of organ preservation times would enable the establishment of global organ matching networks, allowing for more rigorous donor matches and ultimately lead to greater transplant outcomes [15]. Thus, it is crucial to investigate organ preservation at the cellular level to gain a further understanding of the mechanisms of injury and design improved protocols for better

outcomes. In this study, we explored the effects of hypothermic storage at 4 °C and −4 °C on cultured primary rat hepatocytes and demonstrate that plated hepatocytes cold stored for 24 h undergo loss of metabolic activity, membrane destabilization, and upregulated caspase 3/7 activity as compared to standard culture.

Our results reinforce the findings of prior studies that similarly observed hypothermic damage in plated hepatocytes following 24 h of storage in UW solution. Stefanovich et al. [27] and Hossein Aghdaie et al. [21] both reported that hypothermic treatment in UW solution for 24 h resulted in significant decrease of albumin secretion. Abrahamse et al. [16] observed a 75 % increase in LDH release and 60 % decrease in metabolic activity from hepatocytes stored in UW solution for 24 h. Previous research has also shown that PEG supplementation during hypothermic storage of hepatocytes can improve survivability [24,32] and LDH release [23,24]. Likewise, multiple studies have reported signs of apoptosis in cold-treated hepatocytes [16,22,18,25,26,33]. However, studies did not observe a significant improvement in post-storage viability when only inhibiting caspase activity during cold storage [20,21], while PEG supplementation with caspase inhibition during storage resulted in significantly higher post-storage viability than cells treated only with PEG during storage [21]. This is consistent with our findings; emricasan treatment during and after storage did not significantly improve metabolic activity and morphology but dual treatment with PEG and emricasan resulted in metabolic activity that is higher than PEG supplementation alone, suggesting contributing effects from multiple death pathways that result in injury following preservation.

Furthermore, previous research has shown that storing suspended hepatocytes at subzero, non-freezing temperatures improve upon the current cold storage standard [29,24,30,28]. Likewise, we observed the metabolic activity of cold-stored hepatocytes following 2 h of recovery is similar to that of the standard culture along with the PEG-treated SZ-stored hepatocytes having the highest metabolic activity, but after 24 h of recovery, only the combined treated SCS-stored hepatocytes showed comparable metabolic activity to standard culture. This discrepancy suggests that 2 h of recovery is insufficient for cell stabilization following cold storage and reflects the initially high oxygen consumption commonly seen during the recovery of cold-stored whole livers that is correlated with greater subsequent damage, termed ischemia reperfusion injury and mainly attributed to the generation of ROS [34,35,14]. Lower storage temperatures theoretically reduce metabolic activity and cellular processes that could induce cell injury. However, our results show that adherent hepatocytes stored for 24 h at

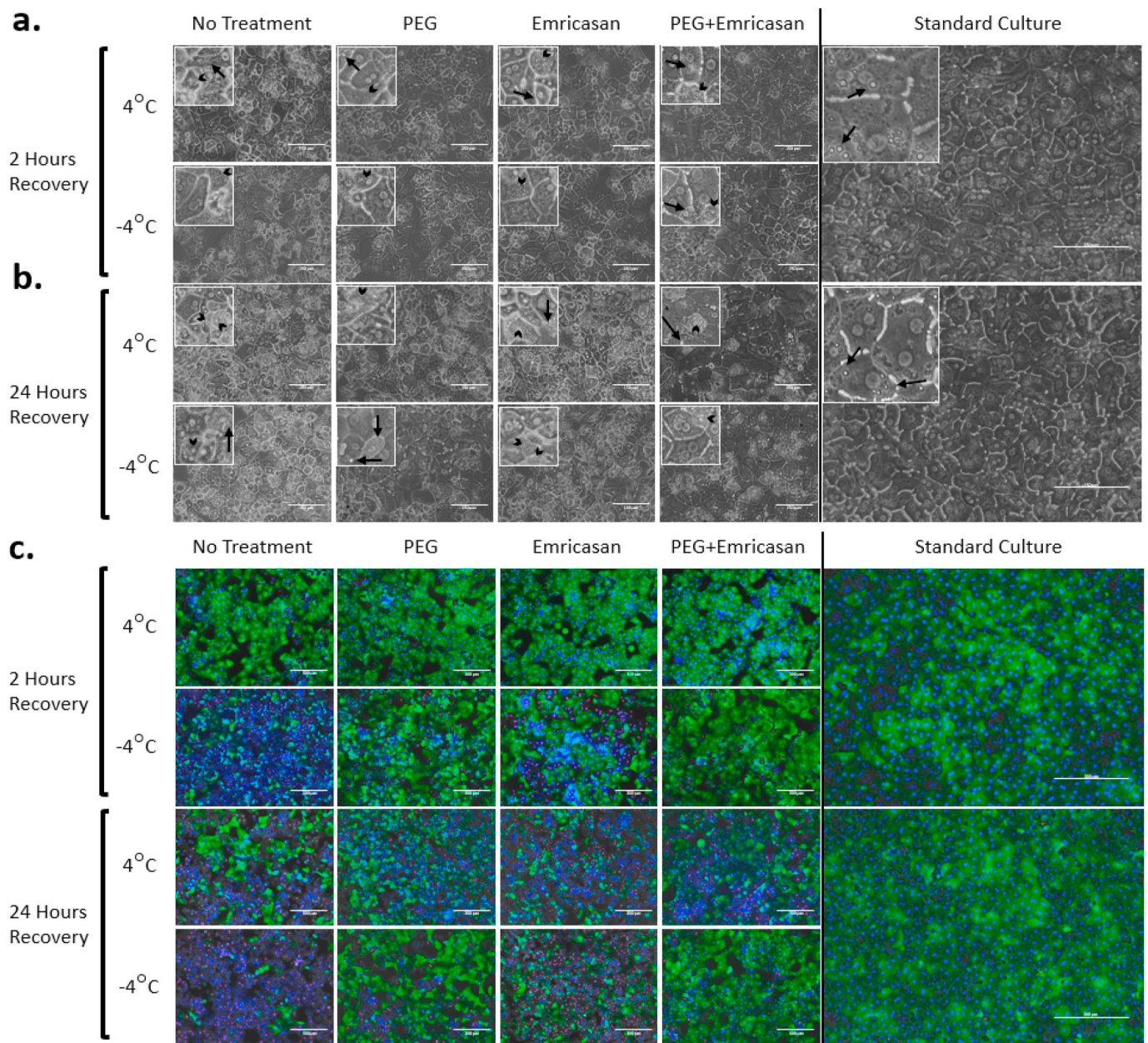


Fig. 6. Morphology of hepatocytes 2 and 24 hours following recovery from storage. Representative phase-contrast microscope images of fresh hepatocytes and cold stored hepatocytes at (a) 2 h of recovery following cold storage and (b) 24 h of recovery. The images shown are representative of three experiments with hepatocytes from different animals. Note the vacuolization (arrows), as well as nuclear collapse (arrowheads) especially after cold storage. 20x magnification. Scale bars: 150 μ m. (c) Representative merged fluorescent microscope images of Live (green)/Dead (red) and nuclei (blue) of the cultures following 24 h of recovery. 10x magnification. Scale bars: 300 μ m. (n = 3 biological replicates). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

subzero, non-freezing -4°C leads to lower cell viability than SCS at 4°C following 24 h of post-storage recovery. This could be attributed to 24 h being an insufficient amount of storage time to induce a beneficial effect and that longer storage periods at -4°C may yield different results. It should be considered that cell attachment and interactions mediated by transmembrane proteins may change the characteristics of cell phenotype, such as gene expression and cytoskeletal structure [36], possibly leading to suspended hepatocytes reacting differently to hypothermia than attached hepatocytes and warranting further investigation. In addition, previous research has reported multiple modes of cell injury from different hypothermic storage temperatures in multiple cell lines [37,38], which we hypothesize to also be the case for cultured hepatocytes and will be further explored in future studies.

While we observed no significant differences between the caspase 3/7 activity of non-treated SCS and SZ groups, PEG-supplementation alone resulted in a significant increase in the caspase 3/7 activity between the SCS and SZ groups following 2 h of recovery, suggesting that PEG-treated cells continue caspase upregulation and that additional mechanisms are leading to caspase upregulation with a decrease in storage temperature. Damages to the mitochondria could offer one possible explanation. As the initiator of the intrinsic apoptosis pathway and subsequent caspase activation, the mitochondrion has been commonly observed to undergo a loss in mitochondrial membrane potential and an increased permeability of the inner mitochondrial membrane during ischemia and reperfusion [39,40,26], leading to mitochondria rupture, the release of cytochrome-c, and activation of the caspase cascade. It is

worth considering that the 35-kDa PEG used in this study may be incapable of crossing the plasma membrane due to its size [41,24,27], with evidence suggesting the protective role of 35k-PEG is localized at the cell surface [41,27]. These findings collectively suggest that our observed increase in caspase upregulation in PEG-treated cells is due to mitochondrial damage.

Building off the results from this study, our future investigations will aim to elucidate additional death pathways and mitochondrial changes that may also concurrently occur from non-freezing storage at multiple subzero temperatures. It is worth noting that our study only addressed apoptosis and cell membrane damage and did not account for other types of hepatic cell death that may also occur [42,35,37,38,43,44,45]. Ferroptosis, which can be the result of lipid peroxidation and has been shown to be inhibited by iron chelator and PEG treatment [46,47,48,22,25,26], could explain the cell membrane damage that we have observed in this study and motivates us to further explore PEG's anti-ferroptotic effects in our future studies. Necroptosis, the regulated form of necrosis in the presence of caspase inhibitors, has been observed during hepatic ischemia-reperfusion through the receptor-interacting protein kinase 3 (RIPK3) pathway [43]. Additionally, Gotoh et al. have reported that autophagic death occurs 15 min into warm reperfusion of liver grafts, with liver dysfunction attenuated by autophagosome inhibition [42]. There is still much to explore of the various molecular pathways that lead to hypothermia-induced upregulation of different modes of cell death such as apoptosis, ferroptosis, necroptosis, and autophagy, in order to identify specific therapeutic targets to improve preservation outcomes [44,45]. Furthermore, it has yet to be explored the long-term effects of an irreversible apoptotic inhibitor, such as emricasan, on hepatocyte function. Nevertheless, our results from this study in combination with the current literature show that cell survival and death during and following preservation is a multifaceted process with multiple death pathways affecting hepatocyte viability. In future studies, we will continue to investigate the confounding temporal effects caused by different mechanisms of hypothermic injury and further optimize preservation solutions by screening not only for biochemical additives, but also for physical variables such as storage temperature.

In summary, we here show that primary rat hepatocytes experience membrane destabilization following both static cold storage and subzero nonfreezing storage and suffer from apoptosis during recovery. We reinforced previous findings of PEG-supplementation during preservation and introduced pan-caspase inhibitor emricasan as an additional supplement to preserve hepatocyte viability and functionality. The results from this study provide a steppingstone to the development of novel hepatocyte specific preservation solutions for increasing cell health and viability following preservation.

Data availability

The datasets generated for the current study are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Huyun Chen: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Bradley W. Ellis:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Antonia T. Dinicu:** Investigation, Writing – review & editing. **Mohammadreza Mojoudi:** Investigation, Writing – review & editing. **Benjamin T. Wilks:** Investigation, Writing – review & editing. **Shannon N. Tessier:** Funding acquisition, Supervision, Writing – review & editing. **Mehmet Toner:** Funding acquisition, Writing – review & editing. **Korkut Uygün:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing, Project administration. **Basak E. Uygün:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of competing interest

Some authors declare competing interests. Drs. B.E. Uygün, K. Uygün, S.N. Tessier, and M. Toner have patent applications relevant to this study. Drs. B.E. Uygün, K. Uygün, S.N. Tessier, and M. Toner have a financial interest in and serve on the Scientific Advisory Board for Sylvatica Biotech Inc., a company focused on developing high subzero organ preservation technology. Competing interests for MGH investigators are managed by the MGH and MGB in accordance with their conflict-of-interest policies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cryobiol.2024.104926>.

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