

CPA toxicity screening of cryoprotective solutions in rat hearts

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ABSTRACT

In clinical practice, donor hearts are transported on ice prior to transplant and discarded if cold ischemia time exceeds ~5 h. Methods to extend these preservation times are critically needed, and ideally, this storage time would extend indefinitely, enabling improved donor-to-patient matching, organ utilization, and immune tolerance induction protocols. Previously, we demonstrated successful vitrification and rewarming of whole rat hearts without ice formation by perfusion-loading a cryoprotective agent (CPA) solution prior to vitrification. However, these hearts did not recover any beating even in controls with CPA loading/unloading alone, which points to the chemical toxicity of the cryoprotective solution (VS55 in Euro-Collins carrier solution) as the likely culprit. To address this, we compared the toxicity of another established CPA cocktail (VEG) to VS55 using *ex situ* rat heart perfusion. The CPA exposure time was 150 min, and the normothermic assessment time was 60 min. Using Celsior as the carrier, we observed partial recovery of function (atria-only beating) for both VS55 and VEG. Upon further analysis, we found that the VEG CPA cocktail resulted in 50 % lower LDH release than VS55 ($N = 4$, $p = 0.017$), suggesting VEG has lower toxicity than VS55. Celsior was a better carrier solution than alternatives such as UW, as CPA + Celsior-treated hearts spent less time in cardiac arrest ($N = 4$, $p = 0.029$). While we showed substantial improvement in cardiac function after exposure to vitrifiable concentrations of CPA by improving both the CPA and carrier solution formulation, further improvements will be required before we achieve healthy cryopreserved organs for transplant.

1. Introduction

In 2019, ischemic heart disease accounted for 550,000 deaths in the United States, making this disease the leading cause of years of life lost [1]. The most durable destination therapy for patients with heart failure is heart transplantation, and the number of these procedures has risen by approximately 50 % between 2008 and 2018, with over 3000 heart transplants performed annually in the United States. As outcome metrics such as 1-year mortality continue to improve [2], there is increasing demand for transplantation, which is increasing an already troublesome balance of supply and demand for donor organs. One of the fundamental challenges to increasing donor organ utilization and increasing equitable access to hearts for transplant is the short acceptable preservation time between recovery and implantation.

In 1990, all surveyed transplant centers used static cold storage (0–

7°C) to preserve donor hearts [3], where the donor heart is flushed with a cold cardioplegic solution and then kept ischemic in that cardioplegic solution during transport. That approach restricts acceptable heart preservation limits to 4–5 h [4]. To overcome that limitation, several new technologies have been examined to increase preservation limits. For example, in normothermic machine perfusion (NMP), donor hearts are perfused with oxygenated blood or blood substitute at body temperature (34–37°C). By delivering oxygen during transport, ischemic damage is minimized, and the organ function can be assessed prior to implant. As of 2015, one NMP device had entered clinical practice (Organ Care System (OCS)) and was used to preserve and assess donor hearts before transplant. Since then, its use has rapidly expanded, with some considering OCS to be the standard of care for marginal donors [5]. Using the OCS, preservation time can be extended to at least 6 h [6]. However, even with these new technologies, preservation times are

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typically limited to 5–10 h [7].

In contrast to the modest increases in organ preservation with NMP, organ cryopreservation has the potential to extend organ preservation time indefinitely. As highlighted by a 2017 *Nature Biotechnology* consensus article, there are numerous benefits to extending preservation times [8]. It is known, for example, that undersized donor hearts are associated with an increased risk of 1-year mortality [9], yet in some cases, these hearts are transplanted anyway due to severity of heart failure and short matching windows. Extending preservation time would ensure that the donor heart is a better match for the recipient. Another benefit pertains to the developing field of immune tolerance induction, in which the goal is to induce the recipient's immune system to tolerate the foreign donor organ. This is in contrast to the conventional approach of immunosuppression, which has adverse cardiovascular side effects [10]. Further, immune tolerance induction is associated with fewer infectious complications [11]. Cryopreservation would allow for a lengthier time period for tolerance induction protocols and allow enough time to evaluate the success of the protocol before commencing the transplant [8]. This elongated transplant schedule may be better for the health of clinicians as well. Nighttime shift work is associated with adverse health outcomes for clinicians, including an increased risk of myocardial infarction [12]. Unfortunately, under the conventional transplant timetable constrained by cold static storage, approximately 50 % of all heart transplants occur at night [13]. Finally, extending storage times may provide a direct benefit to the size of the donor pool. There is a large gap between the number of potential organs available for transplant and the number of those ultimately accepted for transplant, with the 2018 donor heart usage rate at approximately 50 % [2]. In a recent review, prolonged expected cold storage time is given as one of the reasons why donor organs are declined [14].

The challenge in realizing the promise of organ banking is in developing a cryopreservation procedure that does not irreversibly damage the donor heart. One source of damage is ice formation during cooling and/or rewarming. Vitrified cryopreservation offers a potential solution by rapidly cooling the organ to avoid ice formation and storing it at cryogenic temperatures ($\sim -150^{\circ}\text{C}$) in a glassy state (i.e., vitrified). Further, by perfusing the heart with silica-coated iron oxide nanoparticles prior to cryopreservation and then rewarming the heart with a radio frequency coil (i.e., nanowarming), ice formation during rewarming may be avoided [15]. In prior studies by our group and others, the approach of vitrification and nanowarming was used to cool and rewarm rat hearts [16,17]. By perfusing the rat hearts with a high concentration of cryoprotective agents (CPAs) before cooling, ice formation was successfully avoided. However, while some degree of electrical function was preserved [17], the rat hearts did not recover mechanical function during normothermic assessment. Importantly, rat hearts exposed to only CPA perfusion (no cryopreservation) did not recover any mechanical function either, leading us to suspect that chemical toxicity from the 8.4 M cryoprotective solution (VS55 in Euro-Collins) was the primary source of damage.

In the literature, there do exist some CPA screening studies for rat hearts, although the tested CPA concentrations were relatively low. For example, one study reported that ethylene glycol is better than glycerol at a CPA concentration of 2.1 M [18], and another study reported that both are similar at a CPA concentration 0.4 M [19]. Historically, the interest in this field has been in heart preservation by freezing or supercooling ([CPA] < 3 M) as opposed to heart preservation by vitrification ([CPA] > 8 M). Since our preservation approach is vitrification, we are interested in screening CPA cocktails at vitrification-relevant concentrations ([CPA] > 8 M).

In this study, we screened various high-concentration cryoprotective solutions to decrease CPA toxicity. Cryoprotective solutions are made by dissolving an organic CPA cocktail (e.g. VS55) in an aqueous carrier

solution (e.g. EC). VEG is a vitrification-relevant CPA cocktail that has been developed as an alternative to VS55, and resulted in significantly reduced toxicity in kidney slices [20]. We therefore hypothesized that VEG would result in reduced toxicity for rat hearts as well.

Here we show that carrier solution had a substantial impact on the recovery of mechanical function and cell viability. By switching the carrier solution from EC to Celsior, we achieved partial recovery of mechanical function (atria-only beating) after exposure to vitrification-relevant concentrations of CPA. We also show that VEG is superior to VS55 according to the injury metric of LDH release, though VEG did not result in improved functional recovery as compared to VS55.

2. Methods

2.1. Cryoprotective solutions

1. Selection for toxicity screening

Cryoprotective solutions are composed of a CPA cocktail and an aqueous carrier solution. In this study, the CPA cocktails chosen for screening were Vitrification Solution 55 (VS55) and Vitrification Solution with Ethylene Glycol (VEG) (Fig. 1A). VS55 was developed for kidney vitrification and has been used in prior kidney and heart vitrification work [16,17,21,22]. VEG was developed by replacing the propylene glycol in VS55 with ethylene glycol (EG) and has been used in prior kidney vitrification work [20]. In this study and in our prior work, we used a CPA cocktail that contained DMSO as opposed to a CPA cocktail that contained glycerol. One reason for this choice is that glycerol has a lower permeability than DMSO [23]. A high permeability is desirable both for reducing CPA exposure time and for limiting cell volume excursions. Cell swelling can lead to different mechanisms of cell damage including membrane disruption, which has been shown to be significant in other cardiac systems in past studies [24]. We also tested EG-Sucrose, but it led to worse functional recovery and is not described here (Supplemental).

In this study, the carrier solutions chosen for screening were: LM5 [26], Euro-Collins (EC) [27], University of Wisconsin (UW) [28], and Celsior [29] (Fig. 1B). LM5 and EC have been used in prior kidney vitrification work [20,22]. UW and Celsior have been used clinically as cold static storage solutions for human heart transplantation [30]. An overview of the broad categorical differences in carrier solutions is shown in Fig. 1C. Celsior is classified as a hyperkalemic cardioplegic solution because its potassium concentration (15 mM) exceeds that of blood (4.4 mM). UW and EC are classified as intracellular-type solutions because they have a potassium concentration that mimics that of the cytosol (>100 mM). LM5 is classified as a low-salt solution because its total concentration of sodium and potassium (45 mM) is much lower than that of blood (140 mM). Both EC and LM5 contain glucose, whereas Celsior and UW do not. Celsior contains histidine as a buffer, whereas UW, EC, and LM5 contain phosphate as a buffer. Celsior, UW, and LM5 also contain various antioxidants and metabolites whereas EC does not. More information on the rationale behind including certain components over others is provided in the discussion.

It is important for a CPA toxicity screening study to ensure that the vitrifiability (i.e. critical cooling rate) of each screened solution is similar. Vitrifiability may be described as a function of weight by volume percent (w/v %) CPA concentration [31]. We chose a CPA concentration equivalent to that of VS55 (55 w/v %) because simulation studies indicate that rewarming rat hearts faster than the critical warming rate is achievable when using VS55 as the CPA [32]. Note that, although the total molar concentrations of VS55 and VEG are different (Fig. 1A), the w/v % concentration of VEG and VS55 are equal (55 w/v %).

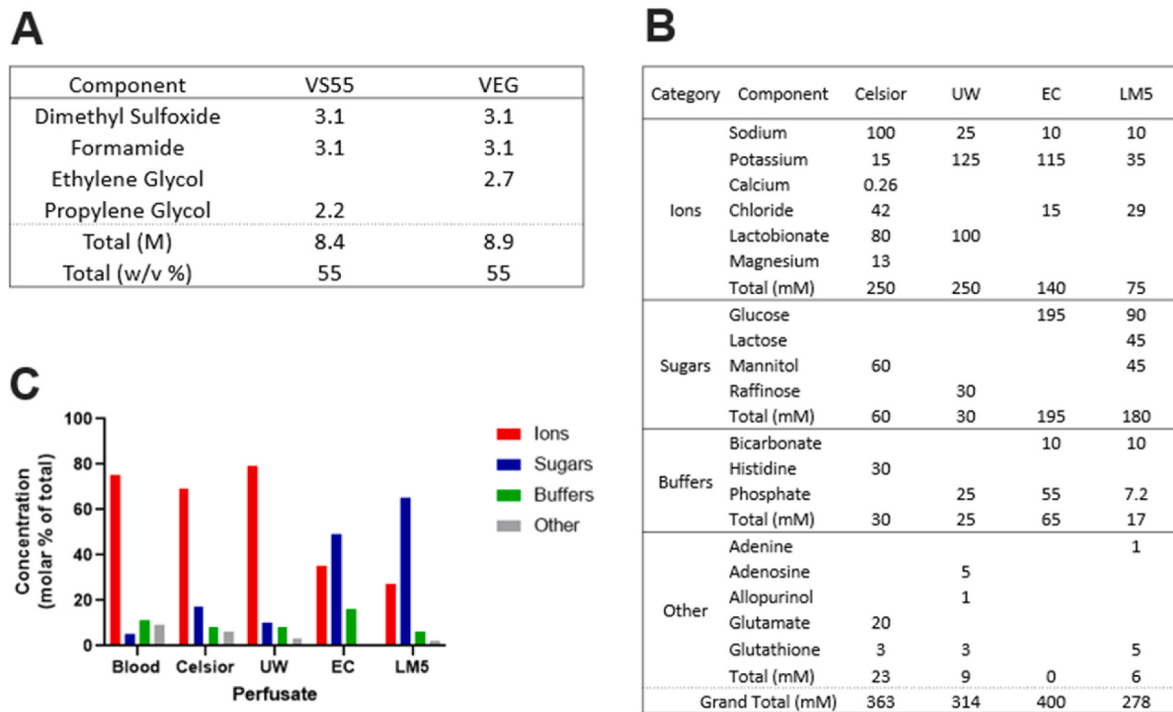


Fig. 1. Compositions of CPA solutions tested. **A:** Compositions and concentrations of CPA cocktails (VS55 and VEG) with units of M for each component. **B:** Compositions and concentrations of carrier solutions (Celsior, UW, EC, and LM5) all with units of mM. **C:** Breakdown of each carrier by molar percent of each listed category in (B), with comparison to blood [25].

2. Preparation

We prepared the CPA and carrier solutions as previously described [33]. Briefly, the appropriate volume of concentrated carrier solution was first added to a volumetric flask. Next, HEPES was added to a final concentration of 10 mM, and a magnetic stir bar was used to mix. Note that the carrier solution does not contain HEPES, only the cryoprotective solution. Then each of the components of the CPA cocktail were added while mixing. Finally, MilliQ water was added until the appropriate volume is reached (e.g. 1 L). The pH was then adjusted to a value of 8 [34]. As a typical example, for 1 L of solution this adjustment involved the addition of 1.8 mL of 5 M NaOH. If the carrier was UW, then a 4:1 KOH:NaOH solution was instead used to increase the pH. Otherwise, the pH was increased with NaOH. If the carrier was UW, then potassium phosphate was used to reduce the pH. Otherwise, the pH was reduced with HCl. After pH-adjustment within 0.1 units, the cryoprotective solution was vacuum-filtered through a nylon membrane (0.2 μ m) to remove particulates. Similarly, carrier solutions and the normothermic perfusate (Tyrode's) were vacuum-filtered through a polyethersulfone membrane (0.2 μ m) the day of perfusion.

2.2. Rat heart procurement

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Minnesota (IACUC Protocol: 2204-39970A). Male Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) aged 10–12 weeks old and weighing 350–400g, were used in this study. The rat heart procurement and cannulation were modified from what we previously described [17]. Briefly, rats were anesthetized with 4 % isoflurane and 1 L-per minute oxygen. The depth of anesthesia was confirmed by toe pinch reflex. The thoracic hair was shaved and disinfected with betadine solution followed by 70 % ethanol. The rats were heparinized (500IU) prior to the incision. The thorax was opened, and the suprahepatic vena cava (SHVC) was clamped. The thoracic aorta was cut, and the heart was

immediately flushed with 20 mL of cold carrier solution mixed with 500IU of heparin through the SHVC. The flushing pressure was not measured. For the case of Celsior as the tested carrier solution, Celsior was used for the flush. For all other tested carrier solutions, UW was used for the flush. Once the flush was done, the heart was procured and cannulated via the ascending aorta to prepare for Langendorff-mode perfusion [35]. The heart was additionally flushed with 10 mL of carrier solution with 250 IU heparin into the cannula. Finally, the heart was weighed to measure the wet mass before perfusion.

2.3. Rat heart perfusion

1. Hypothermic perfusion

The heart was transported in carrier solution on ice ($\sim 4^{\circ}\text{C}$) from the surgery room to the hypothermic perfusion system (15 min ischemic time). Hypothermic perfusion was performed with a previously described multi-thermic perfusion system (Fig. 2) [17,22]. Flow was commenced and computer-controlled via a LabVIEW (National Instruments) interface and the heart was connected while submerged in the organ bath to prevent the entry of air bubbles. A typical flow rate upon connection was 1 mL/min (at ~ 15 mm Hg perfusion pressure). During hypothermic perfusion, the setpoint pressure was ramped up from 0 mm Hg to 50 mm Hg during the first 7 min, then maintained at 50 mm Hg for the remainder of the perfusion. Note that we measured a substantial level of edema after hypothermic perfusion (Fig. S5). A perfusion pressure of 50 mm Hg was chosen for comparison to previous studies [36], though others have identified that lower pressure may be better for preventing edema [37]. During hypothermic perfusion, a proportional-integral (PI) process control algorithm was used to maintain the perfusion pressure to the target value. The input to the controller is an in-line pressure transducer (PendoTECH, PRESS-S-000) upstream from the heart. The PI controller then adjusts the total flow-rate (the sum from both the cryoprotective solution pump channel and the carrier solution pump channel) to ensure that the perfusion pressure

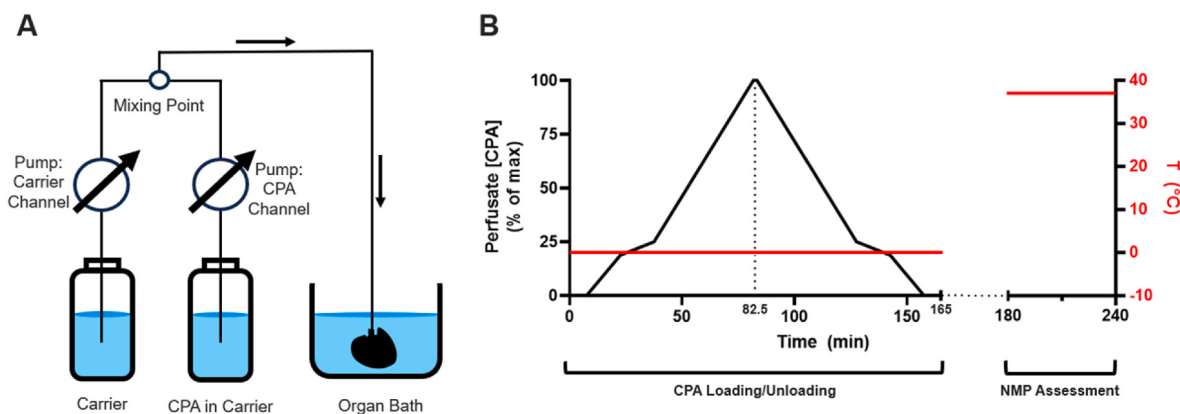


Fig. 2. Overview of CPA perfusion setup and protocol in rat hearts. **A:** Schematic of hypothermic perfusion system. **B:** Perfusion concentration and temperature profiles. First, during hypothermic machine perfusion, rat hearts are loaded/unloaded with CPA. Second, during normothermic machine perfusion (NMP), rat hearts are assessed for recovery of mechanical function.

is at the desired value. Since the pressure transducer was located 20 cm vertically above the heart (equivalent to 15 mm Hg) a setpoint of 35 mm Hg was used to control the heart perfusion pressure at 50 mm Hg.

Separate pump channels were used for carrier and 100 % CPA supply which were then combined in a mixing chamber to achieve desired CPA concentrations. To prevent osmotic damage, the heart was loaded to the desired CPA concentration in a gradual manner. This was accomplished by gradually increasing the flowrate from the CPA channel (Fig. 2A). At the beginning of loading (7.5 min), 100 % of the flow originated from the carrier channel. During CPA loading, the flowrate from the CPA channel was gradually increased until 100 % of the flow originated from the CPA channel at 81.7 min. CPA unloading (from 83.3 min to 157.5 min) was the reverse of this process.

The concentration and temperature profiles are shown in Fig. 2B. The concentration profile chosen for this CPA toxicity screening study was modified from a profile used for prior heart vitrification work (Fig. S1) [17]. The perfusion temperature was maintained at 0°C, and the total hypothermic perfusion time was 165 min. Further details are available in Supplemental Information. After hypothermic perfusion, the heart was weighed (g wet), then placed in the same bottle and storage solution that was used for the initial transport.

2. Normothermic perfusion

The heart was then transported on ice (~4 °C) to the normothermic perfusion system (15 min ischemic time). The normothermic perfusate was modified Tyrode's, whose composition is as follows: 130 mM NaCl, 1.8 mM CaCl₂, 4 mM KCl, 1 mM MgCl₂, 1.2 mM NaH₂PO₄, 24 mM NaHCO₃, and 5.5 mM D-glucose [38–40]. 10 mM HEPES was added to this solution to provide extra buffering capacity, and the CaCl₂ was added last to avoid precipitation. The pH was adjusted to 7.3 with NaOH, and then filtered. The bottle of Tyrode's was oxygenated with carbogen (95 % O₂ and 5 % CO₂) for at least 30 min prior to start of perfusion and remained oxygenated throughout the course of normothermic perfusion via an in-line oxygenator (Radnoti, #130144).

At the beginning, the flowrate was set to 3 mL/min and the heart was connected while submerged in the organ bath to prevent the entry of air bubbles. In this system, the pressure was controlled to 70 mm Hg by manually adjusting the peristaltic pump (Masterflex, #77201-60) flowrate. A value of 70 mm Hg was chosen because this is a common value for *ex situ* rat heart perfusion via the Langendorff method [35,41]. Included in the line of flow is an oxygenator (fed by a second carbogen line), bubble trap, heat exchanger, and pressure transducer. The perfusion temperature was controlled to 37°C via the heat exchanger and a recirculating heater (Cole-Palmer, #MX065135-C11B). In testing, it was found that a heater temperature of 38.5°C yielded an in-line temperature

of 37°C for our system again due to ambient losses. After 60 min of normothermic perfusion, the heart was weighed (g wet) and then placed in a TTC solution to measure cell viability, as described below.

After each run, the perfusion system was cleaned by running first air, then diluted Liquinox, then MilliQ water, and then air through the tubing. Before each run, the perfusion system was sterilized by running 70 % ethanol, then air, then MilliQ water through the tubing.

2.4. Assessments of CPA toxicity

1. Mechanical function

The presence or absence of heart contractions was assessed visually during the 60 min period of normothermic perfusion. To ensure that any visualized beating was the result of myocardial contraction and not peristaltic flow from the pump, the heart was also assessed visually for a brief period after normothermic perfusion (when the pump was stopped). Results were assessed qualitatively according to three categories. A result of 'none' indicates that there was no contraction observed at any time during the 60 min interval. A result of 'atria-only' indicates that there was atrial contraction at any time during the 60 min interval, but no ventricular contraction was observed. A result of 'atria + ventricles' indicates that the entire heart contracted at any time during the 60 min interval. Data were also recorded on the time of first contraction and the duration of contraction.

2. Cell viability

a) TTC staining

TTC staining was conducted in a similar manner to prior work [42]. After normothermic perfusion, the heart was placed in a triphenyl tetrazolium chloride (TTC) solution that was pre-warmed to 37°C. This TTC solution was made by adding 10 mL water, 0.0788 g Trizma Base, 0.213 g Trizma HCl, 0.1 g TTC, and then mixing in a 50 mL tube. Prior to immersion in the TTC solution, the heart was bisected to allow for staining of the endocardium. The heart was kept at 37°C for a period of 15 min, then imaged immediately afterward. The Live Control was taken by staining a fresh control NMP heart. The Dead Control was taken by staining a heart that underwent CPA perfusion (VS55 in EC) at a relatively high hypothermic perfusion temperature (10 °C).

b) LDH release

During the course of perfusion, aliquots were collected from the organ bath for later analysis of lactate dehydrogenase (LDH) content. During the 165 min of hypothermic perfusion, aliquots were collected at 0 min, 25 min, and every 20 min thereafter. During the

60 min of normothermic perfusion, aliquots were collected at 10 min, 30 min, and 50 min. At each time point, three 150 μ L aliquots were collected for $n = 3$ technical replicates. Each aliquot was pipetted into a 0.6 mL microcentrifuge tube, placed on ice during transport, and stored at -80°C until analysis.

Flash-frozen samples were analyzed using the LDH-Glo Cytotoxicity Assay (Promega, cat. J2380). Samples were equilibrated to room temperature and diluted using the recommended LDH Storage Buffer [200 mM Tris-HCL (pH 7.3), 10 % Glycerol, 1 % BSA] with dilutions ranging from 1:2 to 1:500. Samples were assayed in triplicate in 384-well white plates using equal volumes of diluted sample and LDH Detection Buffer. The plates were incubated dark and read on a BioTek Synergy 2 luminescent plate reader at 30 min and 60 min timepoints. The concentration of LDH was determined by reference to a standard curve run on the same plate.

Our reported LDH values were calculated to account for the organ bath volume and inflow/outflow (see further details in Supplemental). To account for heart-to-heart variability in mass, these LDH data were all normalized by mass (g wet before). Note that the reported units here are in U, not mol, in accordance with the activity measurement of the LDH assay.

c) Statistics

$N = 4$ biological replicates unless specifically noted otherwise. Statistical significance is indicated with asterisks: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. All error bars are standard deviations. For normal (or near-normal) group comparisons, T tests and pairwise comparisons using T tests with pooled standard deviation were performed using R (R Foundation for Statistical Computing). For non-normal group comparisons, the Mann-Whitney test was performed using GraphPad Prism (GraphPad® Software, Inc.).

3. Results

In this study, we tested two different CPA cocktails and four different carrier solutions. To avoid testing all the combinations, a single carrier solution was chosen for comparing VS55 to VEG. In prior work, EC was used as the carrier solution. Yet in initial testing, EC-treated hearts did not recover any mechanical function following exposure to 100 % CPA (Fig. S2). We therefore compared VEG to VS55 using Celsior as the carrier. Celsior is a reasonable choice because it was developed for use in the heart [29], and has shown promise in rat heart preservation by

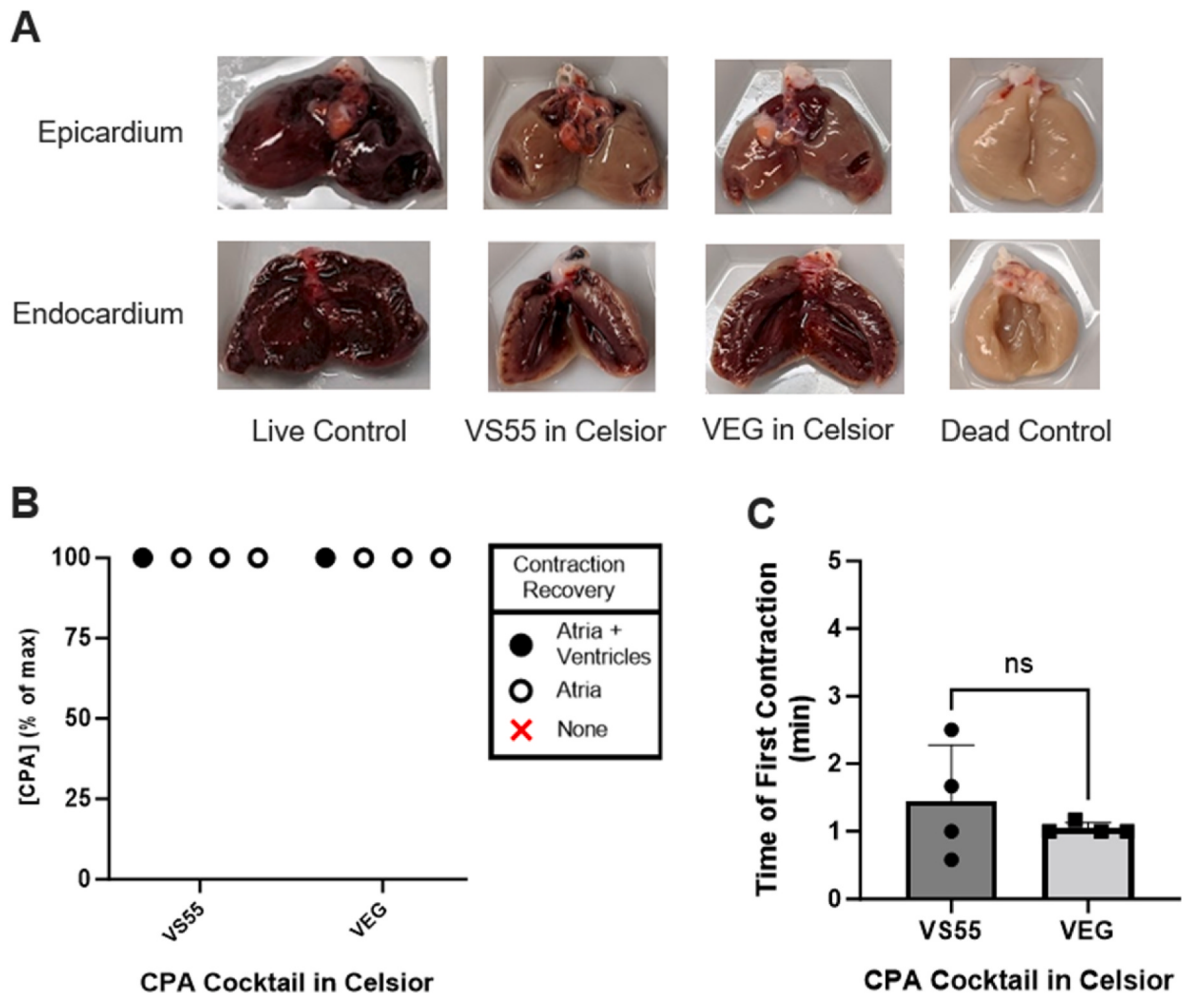


Fig. 3. Rat heart TTC staining and functional recovery after CPA loading/unloading using Celsior as the carrier. **A:** TTC staining of rat hearts following CPA loading/unloading and normothermic perfusion. These images for VS55 and VEG correspond to the best-recovered heart for each treatment group as shown in (B). A dark red color indicates high cell viability whereas a pale color indicates low cell viability. The Live Control is given by a fresh-perfused control heart. The Dead Control is given by CPA perfusion (100 % VS55 in EC) at high temperature (10°C). Note that some tissue is missing from the epicardial surface as the result of procurement of biopsies for later analysis. **B:** Recovery of mechanical function during normothermic perfusion. A CPA concentration value of 100 % indicates that the perfusate CPA concentration reached 55 w/v %. **C:** Time to recovery of atrial beating during normothermic perfusion. Celsior was used as the carrier solution in all cases. Each data point represents one heart. Error bars give the standard deviation. The difference between the two medians (1.3 min vs 1.0 min) was not statistically significant ($p = 0.771$, Mann Whitney test). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

hypothermic perfusion (no CPA) [43].

We found that VS55 in Celsior resulted in similar staining to VEG in Celsior by TTC staining, a qualitative measure of cell viability (Fig. 3A). The displayed images are for the case of perfusion with a vitrification-relevant CPA concentration (55 w/v %). For both VS55 and VEG, the endocardial surface of the heart appeared similar to that of the Live Control. In contrast, the epicardial surface exhibited a cell viability between that of the Live Control and the Dead Control. This partial cell viability corresponds with the partial recovery of mechanical function.

VS55 in Celsior and VEG in Celsior also performed similarly based on recovery of mechanical function. For both VS55 and VEG, all four CPA-treated hearts recovered some mechanical activity. In both cases, three of the hearts recovered atria-only beating, and one heart recovered four-chamber beating (Fig. 3B). For both VS55 and VEG, heart reanimation occurred equally early into the 60-min duration of normothermic perfusion with Tyrode's (Fig. 3C). We also measured vascular resistance and edema, which were similar for both VS55 and VEG (Fig. S5). In all cases, the recovered beating of hearts exposed to 100 % CPA (55 w/v %) was noticeably weaker than that of the no-CPA control hearts. Still, recovering any level of functionality after perfusion with 100 % CPA (55 w/v %) is an important development as it is a necessary but not sufficient condition for successful vitrification and rewarming of a whole heart. To our knowledge, this is the first report of a whole heart recovering any degree of cardiac contractility following perfusion with a vitrification-relevant CPA concentration. Specifically, the former highest concentration reported was 37 w/v% (6 M EG) [44].

In TTC-stained hearts, it was common to observe a pale region of low-viability tissue extending from the epicardium into the myocardial wall (Fig. 3A). For VS55 in Celsior versus VEG in Celsior, this region of low viability was consistently larger for VS55-treated hearts.

We further obtained quantitative data on injury due to CPA toxicity by measuring the concentration of lactate dehydrogenase (LDH) in the organ bath during hypothermic perfusion (CPA loading/unloading) and normothermic perfusion (no CPA). Clinically, LDH release has been used as a biomarker of myocardial infarction [45], with higher levels of LDH in the blood indicating a higher level of injury [46]. During hypothermic perfusion (CPA loading/unloading), we found that LDH release for VS55 and VEG were similar for most time points (Fig. 4A). During normothermic perfusion (no CPA), however, we found that LDH release for VEG in Celsior was lower than that of VS55 in Celsior (Fig. 4B), suggesting that VEG is less toxic. Upon calculating the total LDH released during normothermic perfusion (Fig. 4C), it was found that VEG in Celsior caused a significantly lower level of LDH release than VS55 in Celsior ($p = 0.017$). This difference was not significant when only hypothermic perfusion was considered.

With VEG identified as a less toxic CPA cocktail, we proceeded to further investigate the effect of carrier solutions on cell viability and functional recovery (Fig. 5). At 100 % concentration of VEG % (55 w/v %), we found that Celsior exhibited the best cell viability as observed by TTC (Fig. 5A). For UW, the endocardial surface was stained similarly to that of Celsior and the Live Control groups. However, the epicardial surface was stained worse than that of Celsior, indicating lower cell

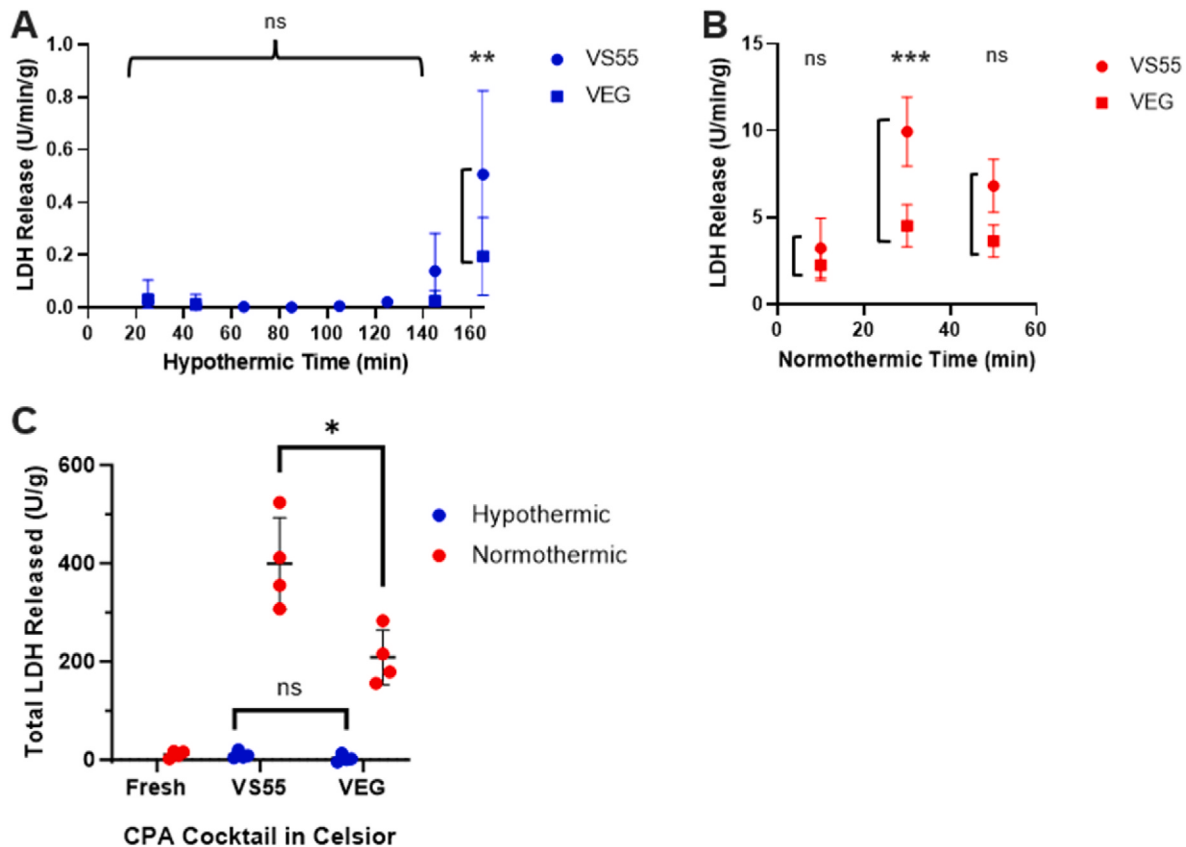


Fig. 4. LDH release during rat heart hypothermic and normothermic perfusion with VS55 and VEG in Celsior. **A:** LDH release rate during hypothermic perfusion. Each data point gives the average of $N = 4$ hearts. Shown above are the pairwise comparisons (T tests with pooled standard deviations) for VS55 versus VEG at the corresponding time points ($p > .05$ for time points below 165 min, $p = 0.006$ for 165 min using Holm-adjusted p -values). **B:** LDH release rate during normothermic perfusion. Each data point gives the average of $N = 4$ hearts. Shown above are the pairwise comparisons (T-tests with pooled standard deviations) for VS55 versus VEG at the corresponding time points ($p = 1.0$ for 10 min, $p < .001$ for 30 min, $p = 0.052$ for 50 min using Holm-adjusted p -values). **C:** Total LDH released during hypothermic perfusion and normothermic perfusion as calculated from (A) and (B). The data are normalized to wet weight before hypothermic perfusion. All error bars give standard deviations of $N = 4$ biological replicates. Each data point represents one heart. The difference between VS55 and VEG was statistically significant for normothermic perfusion ($p = 0.017$, Welch T-test) but not for hypothermic perfusion ($p = 0.240$, Welch T-test).

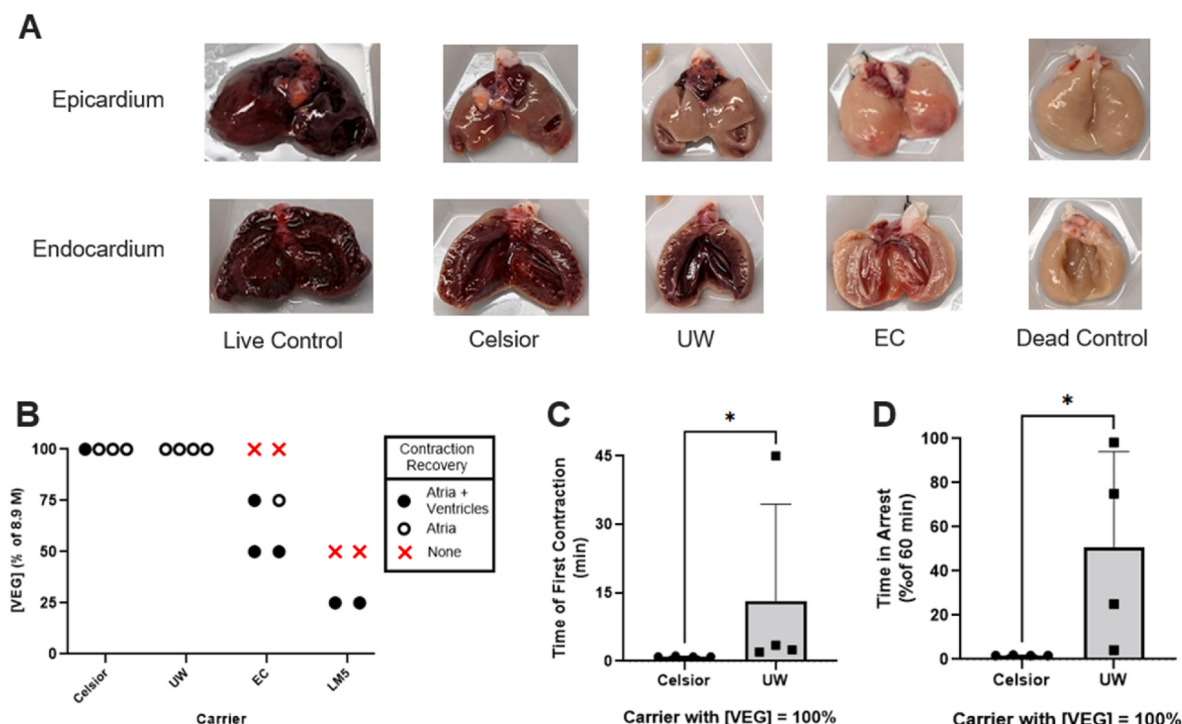


Fig. 5. Rat heart cell viability and functional recovery based on perfusion with VEG and different carrier solutions. **A:** TTC staining of rat hearts following normothermic perfusion after CPA loading/unloading. These images for each carrier correspond to the best-recovered heart as shown in (B). A dark red color indicates high cell viability whereas a pale color indicates low cell viability. The Live Control is given by a fresh-perfused control heart. The Dead Control is given by CPA perfusion (100 % VS55 in EC) at high temperature (10 °C). Note that some tissue is missing from the epicardial surface as the result of procurement of biopsies for later analysis. **B:** Recovery of mechanical function during normothermic perfusion after CPA loading/unloading. A CPA concentration value of 100 % indicates that the perfusate CPA concentration reached 55 w/v %. **C:** Time to recovery of atrial beating during normothermic perfusion. The difference between the two medians (1.0 min vs 3.0 min) was statistically significant ($p = 0.029$, Mann Whitney test). **D:** Time in cardiac arrest (no atrial or ventricular beating), including the time before the first observed contraction. The difference between the two medians (1.7 % vs 50 %) was statistically significant ($p = 0.029$, Mann Whitney test). VEG was used as the CPA cocktail in all cases. Each data point represents one heart. Error bars give the standard deviations. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

viability than Celsior overall. For the case of EC, both the endocardial and epicardial surfaces resembled the Dead Control, indicating that the EC carrier resulted in higher CPA toxicity.

These results correspond with the recovery of mechanical function observed for the groups of different carrier solutions. EC resulted in no visible recovery of beating in the case of 100 % VEG (Fig. 5B). The carrier of LM5, moreover, resulted in no visible recovery of beating even at a VEG concentration of 50 % (27.5 w/v %), indicating that the use of LM5 as a carrier resulted in the greatest degree of injury. Celsior and UW carriers both resulted in partial recovery of beating in the case of 100 % VEG (55 w/v %). We also measured vascular resistance and edema, which were somewhat different depending on the carrier (Fig. S5).

In the case of perfusion with 100 % CPA, the Celsior-treated hearts functioned better than the UW-treated hearts during the 60 min of normothermic perfusion. Celsior-treated hearts recovered atrial beating within 1 min of reperfusion. In contrast, UW-treated hearts took significantly longer (Fig. 5C). In addition, two of the four UW-treated hearts ceased their atrial contractions after an initial period of reanimation. Combined with the longer duration before the start of contraction, this resulted in UW-treated hearts spending a significantly longer time in a state of arrest than Celsior-treated hearts (Fig. 5D). We therefore recommend VEG in Celsior be used for reduced CPA toxicity in future studies.

4. Discussion

Our prior work in rat heart cryopreservation was focused on establishing the feasibility of vitrification and nanowarming by avoiding ice formation during cooling and rewarming. That physical demonstration

was successful, and in addition, the rat hearts recovered some degree of electrical function during normothermic assessment, though they did not recover mechanical function [17]. Here, we report the recovery of mechanical function in a whole heart following exposure to CPA concentrations that can enable vitrification and rewarming. This improvement was achieved by focusing wholistically on the composition of the cryoprotective solution (both CPA and carrier). We identify two key findings.

First, Celsior was a better carrier solution than EC when the cumulative effects of toxicity were considered. Switching to Celsior resulted in substantially improved recovery of mechanical function and cell viability (Fig. 5). This finding is consistent with related research on cold storage of donor hearts (no CPA). In rat hearts, it was found that cold static storage in a precursor solution to Celsior (“Solution I”) produced better left ventricular developed pressure than cold static storage in EC [47]. Similarly, in human right atrial trabeculae, it was found that EC was worse than UW for 24 h of static storage at 12 °C [48]. As for our finding that Celsior is better than UW, this is consistent with some cold static storage results [30], but inconsistent with others [49]. More importantly, our results are consistent with other findings using hypothermic perfusion of rat hearts for storage, in which Peltz et al. found that Celsior preserved the heart slightly better than UW [43]. These cold storage results are therefore broadly consistent with our findings, though care should be taken in making this comparison. For our application of cryopreservation, the addition of high-molarity CPA components likely influences the carrier solution’s performance in these complex conditions.

There are many differences between the four tested carrier solutions (Fig. 1), so it is difficult to clearly ascertain which changes were

responsible for the improved recovery we observed. However, prior studies on DMSO-perfused rat hearts may provide some guidance. When the NaCl in a carrier solution containing 142 mM NaCl was substituted for 142 mM sucrose and 71 mM KCl, contractility decreased from 36 % to 18 % of initial values. This decrease was not observed when the type of sugar was mannitol instead of sucrose [50]. This finding suggests that the type of sugar matters in a carrier solution. It could be the case that certain sugars such as lactose (in LM5) are worse for heart perfusion with VEG, whereas other sugars such as mannitol (in Celsior) are better for heart perfusion with VEG. More studies would be needed to investigate this.

Note that CPA toxicity, not cold ischemia, is expected to be the main source of damage in our studies (Fig. S3). Still, it may be the case that certain compounds are helpful in the heart's response to CPA exposure. There have been several studies underpinning the development of Celsior as a cold storage solution. The following compositional choices in Celsior may have provided some benefit:

(1) Low [K]/[Na] ratio

Celsior contains a relatively low [K]/[Na] ratio because a higher [K]/[Na] ratio is associated with worse myocardial ATP preservation [51]. ATP consumption is accentuated by two mechanisms. In the first mechanism, high [K] (>30 mM) causes voltage-gated calcium channels to open, resulting in the ATP-dependent contraction of vascular smooth muscle and myocardium [52–54]. Of the solutions we tested, only Celsior has [K] < 30 mM. In the second mechanism, low [Na] (e.g. 10 mM) also causes increased contraction, presumably as a result of increased activity of the Na/Ca exchanger [55]. Of the solutions we tested, Celsior had the highest [Na] at 100 mM.

(2) Magnesium

Celsior contains magnesium, whereas, in our preparation, the other solutions do not. Magnesium is known to cause a decrease in [Na]_i [56], which may be helpful in decreasing calcium overload. Among other negative consequences, calcium overload is known to result in ATP depletion [57].

(3) Histidine and pH

Celsior contains histidine instead of other buffering agents such as phosphate or bicarbonate. Phosphate may precipitate in solution when mixed with calcium under certain conditions [41], and therefore there is a concern that phosphate may precipitate with *in vivo* calcium as well. This concern may be particularly salient given a recent study that implicates mitochondrial calcium phosphate precipitation as a key pathological mechanism in calcium overload [58]. As for histidine and bicarbonate, histidine was found to be better than bicarbonate at maintaining intracellular pH at a hypothermic temperature (15 °C). Note, however, that in this same study, bicarbonate produced at least as good levels of creatine phosphate and ATP as histidine. Overall, these authors found that an acidic perfusate pH (pH < 7.4) was better for rat hearts [59], perhaps because calcium uptake is lessened at lower intracellular pH [60]. A key benefit of histidine over bicarbonate, however, is its ROS scavenging capability. Specifically, histidine has been shown to prevent singlet oxygen from damaging the Na⁺/K⁺-ATPase [61]. Note that Celsior also contains lactobionate and glutamate, which also exert some buffering ability.

(4) Glutamate

Glutamate was included based on its role in producing α-ketoglutarate, a substrate for anaerobic ATP generation. When glutamate was added to the perfusate, hearts were able to better maintain ATP levels during a period of hypoxia [62].

(5) Reduced glutathione

Reduced glutathione was included in Celsior in an effort to protect mitochondria from calcium influx, a phenomenon known to occur if the ratio of reduced glutathione to oxidized glutathione decreases beneath a certain threshold [63].

One potential hypothesis may be applied to a simplified understanding of CPA-induced damage: high CPA concentrations may intrinsically or osmotically cause oxidative stress, which in turn causes calcium overload, which in turn causes low ATP. The components of a carrier solution may target any one of these stages. First, components such as histidine and reduced glutathione may address oxidative stress. High ROS levels have been reported in cartilage tissue that was loaded/unloaded in one step with 9 M CPA (DMSO, EG, PG) at 4 °C [64], so it is plausible that high ROS levels are occurring as a result of our ramp exposure to 8–9 M CPA at 0 °C. Second, sodium and magnesium may address calcium overload as presented above. Third, glutamate may address low ATP as presented above. Further testing will obviously be required to better evaluate the potential mechanisms of injury and potential protection.

One future direction is to develop a carrier solution specifically for use in heart. Generally, our results demonstrate that the carrier solution component of the cryoprotective solution, while often overlooked, may have a substantial effect on the cell viability and functional recovery of CPA-exposed tissue. When the carrier was LM5, the toxicity threshold for partial recovery of beating was <50 % VEG. When the carrier was Celsior or UW, by contrast, this toxicity threshold was ~100 % VEG. CPA toxicity is generally considered to be an exponential function of CPA concentration, so this effect of carrier solution on CPA toxicity is quite substantial.

In kidney slices, Clark et al. also observed a substantial effect of carrier solution on viability [65]. To improve kidney cryopreservation outcomes, the Fahy group focused on the development of a kidney carrier solution and achieved great success. By modifying the existing RPS-2 solution [66] into LM5, Fahy et al. achieved substantially improved recovery following CPA loading/unloading. When VS55 was the CPA, LM5 resulted in a 16 % higher viability (81 % versus 65 %) as compared to RPS-2. When VEG was the CPA, LM5 resulted in a 9 % higher viability (99 % versus 90 %) as compared to RPS-2 [20]. LM5 is very similar to RPS-2, merely containing an additional two sugars (45 mM mannitol and 45 mM lactose substituted for 90 mM glucose). It is therefore unsurprising that we observed a substantial effect of carrier solution on recovery when comparing carrier solutions that differed by more than just sugar composition.

Our second major finding was that VEG is a better CPA cocktail than VS55 from a chemical toxicity perspective. Switching to VEG resulted in reduced injury as measured by LDH release (Fig. 4). This finding is consistent with prior results in kidney slices, in which VEG in LM5 resulted in 98 % viability as compared to 81 % for VS55 in LM5. Fahy et al. explain this result with the qv* hypothesis. In brief, the idea is that nonspecific CPA toxicity is the result of macromolecular dehydration. The VS55 cocktail contains PG, whereas the VEG cocktail contains EG. As indicated by its higher value of qv*, PG has a higher hydrogen bonding strength than EG. One would predict, therefore, that PG would result in increased macromolecular dehydration as compared to EG, resulting in increased CPA toxicity. Note that the molar concentration of EG in VEG (2.7 M) needed to be higher than that of PG in VS55 (2.2 M) since, by this same mechanism, PG is a better glass-former and therefore does not require as high a CPA concentration to produce the same effects on vitrification. Fahy's idea, therefore, is that the effect of a high qv* on nonspecific CPA toxicity outweighs its effect on lowering the CPA concentration needed to vitrify, hence VEG is better than VS55 [20]. An alternative explanation is that the specific toxicity of PG at 2.2 M for heart is higher than that of EG at 2.7 M for heart, though it is unknown which specific molecules or types of molecules PG or EG would be damaging. Regardless, further improvements need to be made to recover

cell viability and mechanical function similar to cold storage controls.

In Fig. 4 we presented the dynamics of LDH release during hypothermic and normothermic perfusion. Note that LDH release during normothermic perfusion (no CPA) was one order of magnitude higher than that of hypothermic perfusion (CPA loading/unloading). Of specific interest here is the fact that the time of peak LDH release (midway through normothermic perfusion) did not coincide with the time of peak CPA concentration (midway through hypothermic perfusion). In studies done on closed chest myocardial infarctions in dogs, Wroblewski et al. documented a time lag of ~12 h between the incident of myocardial injury and the measurement of peak LDH content in the perfusate effluent [46]. Therefore, the LDH spike during normothermic perfusion may be a result of cell swelling (inability to control cell volume owing to mitochondrial damage, Na^+ , K^+ -ATPase failure, or increased membrane permeability) caused by the earlier period of toxic CPA exposure at 0 °C. This explanation is consistent with TTC staining results, which show that a more toxic exposure (8.4 M VS55 versus 6.3 M VS55) at 0 °C results in nonviable tissue after a 60 min period of normothermic perfusion (Fig. S3).

The LDH spike observed towards the end of hypothermic perfusion may be a result of osmotic damage (cell swelling) near the end of the CPA unloading process. This is consistent with the observed increase in resistance. In hearts exposed to a higher level of osmotic stress during unloading (step versus ramp CPA unloading), vascular resistance was higher (Fig. S1), suggesting that a high level of vascular resistance may be a result of osmotic damage. This is consistent with what others have reported for kidney CPA perfusion [67]. It is plausible that, although vascular resistance was substantially reduced using ramp versus step unloading, there is still osmotic damage occurring using our ramp CPA unloading profile. Note that a preliminary attempt to resolve this potential problem by lowering the ramp rate (and therefore reducing osmotic stress) was unsuccessful (Fig. S4). Future work may benefit from a thorough study on identifying the osmotic tolerance limits in cardiac tissue.

One other notable observation is that in all studies involving high CPA concentrations (>4 M), the endocardium exhibited higher cell viability than the epicardium by TTC (Fig. 3). As for functional recovery, we observed several cases of atria-only contraction, but no cases of ventricle-only contraction (Fig. 3). Understanding why CPA exposure is having differential effects across the heart will be necessary to develop a successful heart CPA perfusion procedure.

One explanation for higher cell viability in the endocardium is that the endocardium has a higher capacity for anaerobic metabolism. In control biopsies taken from the canine left ventricle, it was found that the endocardium contains a higher level of glycogen and lactate than the epicardium [68]. This indicates that the endocardium has higher anaerobic capacity [69], and therefore may be better able to tolerate a period of ischemic CPA perfusion. Future studies may involve measuring such metabolites in CPA perfused hearts.

In prior studies on DMSO-perfused rat hearts, Offerijns et al. also observed atria-only recovery. In six of eighteen hearts frozen and then rewarmed (ice-allowed preservation at a storage temperature of -30 °C), atria-only recovery was observed. In four of these six hearts, no QRS complex was observed, indicating that the CPA loading and freezing process affected the electrical conduction capability of the ventricles. In the other two hearts, however, a QRS complex was observed. This indicates that, for these hearts, the CPA loading and freezing process affected the excitation-contraction capability of the ventricles. These effects may be a result of DMSO exposure itself, not just the freezing process. When DMSO was loaded at a concentration of 1.4 M or more, an atrioventricular block was observed. Upon CPA unloading, this electrical disruption was reversible at low (<3.5 M) DMSO concentrations but irreversible at high (>4.2 M) DMSO concentrations [70]. Armitage et al. also observed this effect of CPA concentration on ventricular recovery. At 6 M EG, only one of three hearts recovered any contractions following supercooling at -1 °C, and this recovery was

limited to the atria. At 3 M EG, three of four hearts recovered atrial plus ventricular contractions following supercooling at -1 °C [44]. In our study using 8.9 M VEG in Celsior, all four hearts recovered atrial beating, with one heart recovering ventricular beating as well. Future studies may benefit from measuring the electrical activity of CPA-perfused hearts.

Cell viability by TTC did not always correspond with mechanical function (Fig. S6). Whereas TTC staining levels similar to the Live Control or the Dead Control always corresponded to heart beating and heart arrest, respectively, an intermediate level of TTC staining did not always correspond to an intermediate level of beating. In some cases of intermediate staining (VEG in UW), the heart did recover beating prior to being stained. In other cases of intermediate staining (VEG in LM5), the heart did not recover any beating. Tissue stains red ("viable") upon incubation in TTC solution as a result of cytochrome *c* oxidase activity (Complex IV on the mitochondrion) [71]. In the presence of a reducing agent (e.g. NADH), cytochrome *c* oxidase reduces TTC into a red formazan product [72]. Note that it is possible to obtain a misleading TTC result in the case that the tissue has been recently ischemic, as NADH levels and cytochrome *c* oxidase activity have not yet had time to decline. For rat hearts, it has been shown that 60 min of reperfusion following ischemia is sufficient to identify infarcted tissue [73]. Therefore, in our study, all hearts were reperfused normothermic for 60 min prior to staining. As such, it is our interpretation that a positive (red) staining indicates viable tissue. Namely, TTC staining results indicate that certain cryoprotective solutions (VEG in LM5) preserved the NADH levels of the heart but did not preserve other metabolites or structural elements necessary for contraction. One structural element that may not be preserved is t-tubules. In skeletal muscle preparations, glycerol exposure and in particular unloading results in t-tubule destruction, presumably as a consequence of exceeding an upper osmotic tolerance limit in fast versus slow unloading [74,75]. In cardiomyocytes, even a modest degree of cell swelling (1.2 times the initial cell volume) is sufficient to cause substantial detubulation [24]. Therefore, it is plausible that t-tubule destruction occurred in all of our runs, despite the 75 min of gradual ramp unloading. Nevertheless, studies show that cells are able to recover their t-tubule structure in some cases [76]. Among other factors, high ATP content is beneficial for normothermic t-tubule recovery [77]. More work is needed to address the impact of our protocols on t-tubules.

One limitation of this study is that the CPA concentration in the cellular compartment was not measured. In an ideal CPA screening study, the tissue would be loaded to an equivalent CPA concentration (w/v %) since CPA concentration (w/v %) is correlated with vitrifiability [31]. In this study, a perfusate CPA concentration of 55 w/v % was used. This study is limited, though, because the mass transfer rates of the two CPA cocktails may not be identical. It is known that PG has a higher cell membrane permeability than EG Ref. [78]. It is possible, therefore, that the higher level of LDH release for VS55 (Fig. 4) is a result of increased intracellular CPA concentration, not increased chemical toxicity.

The CPA perfusion profile used in this study (Fig. 2B) has not been optimized for achieving sufficient CPA loading with minimal damage. It is the subject of future work to optimize the CPA loading/unloading profile for reduced toxicity as others have done for their systems [79–81]. Still, one positive feature of the profile used in this study is that it involves ramp loading/unloading. In prior work, we used a step profile to load/unload CPA [17]. This step profile may have resulted in an increased level of osmotic damage as compared to the ramp profile used in this study. Therefore, it is plausible that the improvement in vascular resistance as shown in Fig. S1 is the result of switching to a less damaging CPA profile as opposed to switching to a less toxic cryoprotective solution. Further studies would need to be done to characterize the effect of switching from step to ramp.

5. Conclusion

When using Celsior as the carrier, both the VS55 and VEG CPA cocktails led to the consistent recovery of atrial beating in all four replicates and the recovery of four-chamber beating in one of four replicates. LDH release during normothermic perfusion was higher for VS55 in Celsior than for VEG in Celsior, suggesting that VS55 is more toxic than VEG. Celsior produced significantly better functional recovery and cell viability than all other carrier solutions tested, demonstrating that carrier solution may have a substantial effect on CPA toxicity. It may be the case that anaerobic ATP generation during CPA loading/unloading is important for the heart to recover mechanical function, which would explain why the glutamate-containing Celsior fared best in this study.

To develop a carrier solution for successful heart cryopreservation, it may be necessary to better understand the mechanism of CPA toxicity in cardiac tissue and its apparent reduction by carrier solution. This may call for more fundamental experiments in a higher-throughput cardiac system. As such, both the CPA screening and the carrier screening performed in this study may be best viewed as a first step. This is the beginning, not the end, of developing a successful cryoprotective solution for the heart.

Declaration competing of interest

None.

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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.2023.104842>.

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