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Graft preservation

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Graft preservation

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Editorial: Graft preservation

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Editorial on the Research Topic
Graft preservation

Solid organ transplant outcomes have tremendously benefited from innovations in graft preservation strategies (1). *Ex-vivo* (or *ex-situ*) machine perfusion (EVMP) for organ preservation protects donor organs from the injuries traditionally encountered by cold static preservation (CSP), thus maintaining the organs in a "physiologic" state, minimizing rates of primary graft dysfunction (PGD) and promising functional recovery of marginal organs. EVMP has already demonstrated the capability to extend preservation periods well beyond what is considered acceptable after CSP for the kidney, liver, lung, and heart (2). EVMP strategies have expanded the donor pool and pushed the limits of the extended criteria toward donor organs previously excluded from transplantation (3). In this landscape, the use of donation after circulatory death (DCD) organs has been a game- changer that, before the emergence of the normothermic regional perfusion (NRP) technology (4), seemed strictly dependent on the availability of a platform to evaluate the organ *ex-situ*. The potential to recondition organs that could otherwise be discarded represents the "holy grail" of the new frontiers of EVMP (warm or cold). The challenge of extending preservation time and the growing use of EVMP during organ procurement have introduced the potential for administering therapeutics during this period to improve organ quality and mitigate post-transplantation complications. The already hot topic of heart allocation promises to be entirely revolutionized by the perspective of reducing the impact of ischemic time and broadening the area for the allocation, also encompassing immunologic compatibility, to a broader supranational geographic region (North America, Europe, Australia).

The primary objective of this research topic was to collect expert opinions, original research articles (clinical, translational, basic), case reports, and reviews (both brief and expansive) that address critical gaps in knowledge in the field of graft preservation and recent developments in applications of EVMP.

Lechiancole et al. set the stage for this collection by providing an extensive review of the use of various CSP and EVMP strategies in clinical heart transplants. The authors summarized the available clinical data and provided perspectives on technical aspects and limitations to current preservation techniques. Similarly, Iske et al.

provided an interdisciplinary overview of the current abdominal and thoracic EVMP systems and organ-specific preservation protocols and summarized relevant EVMP applications beyond organ preservation for allogeneic transplantation.

Kasinpila et al. described an unusual case of a 55-year-old successfully retransplanted (21 years after the prior) with a DCD donor heart from a distant location, necessitating an extended transport period (>7 h) with normothermic EVMP. This article highlights the imbrication between perfusion technology and the expansion of DCD donation and shows how leading institutions are pushing the limits through EVMP.

There remains an unmet clinical need for a biomarker or a tool to ascertain organ quality during preservation. Mendiola Pla et al. applied video kinematic evaluation (Vi.Ki.E.) and assessed the feasibility of using this method to measure *ex vivo* cardiac kinematics. Porcine donor hearts underwent normothermic EVMP on the TransMedics® Organ Care System (OCS™). Vi.Ki.E. performed while the donor's hearts beat on the OCS™ could be applied to predict cardiac fitness and allow a reliable organ assessment.

Radomsky et al. compared the concentration levels of cytokines/chemokines in different perfusion solutions during *ex vivo* lung perfusion (EVLP) after 1 and 9 h of CSP using a porcine cardiac arrest model. While the concentrations of many inflammatory cytokines increased across all experimental groups, a longer period of CSP before EVLP did not result in an enhanced inflammatory protein secretion into perfusates. This knowledge may define the optimal lung preservation method that could potentially increase the donor lung pool.

A study by Niroomand et al. (from the Lund group in Sweden, which has driven the field of cytokine absorption in EVLP) utilized mass spectrometry-based proteomics and bioinformatics approaches to understand molecular mechanisms of how cytokine absorption impacts lung function when used during EVLP. This study revealed characteristic inflammatory, immunomodulatory, and coagulation pathway differences between the lungs treated with and without cytokine adsorption, which may lead to more targeted approaches to improve lung function.

Vervoorn et al.'s literature review focused on administering gene therapies delivered by EVMP. This review examined 23 studies of gene therapy applied to the heart during both hypothermic and normothermic EVMP conditions, using different vectors, perfusion conditions, duration of exposure to the vector, doses, and perfusion composition. Gene therapy delivered via EVMP has applications in both allo- and auto-cardiac transplantation. Autotransplantation during support with cardiopulmonary bypass may be the "moon-shot" to repair *ex-situ* a heart with a pathogenic mutation.

Ughetto et al. provide a comprehensive review of the mechanisms involved in ischemia- reperfusion injury to the heart during transplantation and existing targeted strategies useful to minimize injury leading to PGD. Treatments reviewed include pharmacological agents, gene therapy, cell therapy, metabolic modulation, and targeted drug delivery, all of which can be provided during EVMP. The most attractive solutions highlighted are blocking apoptosis and necrosis pathways, extracellular vesicle therapy, and donor heart-specific gene therapy.

McCully et al. provided an impressive overview of mitochondrial transplantation's potential as a novel methodology for rescuing cell viability and function following ischemia-reperfusion injury and its potential applications. The thoughtful amount of data supports the versatility and durability of such an approach, stimulating the interest in imbricating this technology with the current standard of graft preservation.

Andrijauskaite et al. utilized a newly developed portable hypothermic oxygenated machine perfusion device (the VP.S Encore) to evaluate unused human donor hearts. After placing these hearts on this innovative and simple-to-use cardiac preservation device for an extended period of time, the authors evaluated cardiac function by placing them in a Langendorff system for reperfusion and evaluation of cardiac contractility. These data constitute a step toward the clinical use of a device warranting hypothermic oxygenated perfusion. Provoost et al. report the first experience with the portable LUNGguard showing short-term outcomes that were safe and beneficial and the possibility of converting the transplant procedure to a diurnal activity. The opportunity to imbricate prolonged cold ischemic time and prolonged perfusion coupled with a reliable possibility of evaluating organ function offers further opportunities to redesign organ transplantation logistics.

Spencer et al. report efforts from the Extracorporeal Life Support Laboratory (University of Michigan) to prolong safe EVMP. Prior studies from this group demonstrated that the metabolic and biological basis for graft failure associated with prolonged EVMP was due to changes in blood composition over the perfusion period. In the current report, incorporating plasma exchange or ultrafiltration to the OCS circuits enabled successful perfusion of 24 h. In contrast, the majority of hearts perfused without these interventions failed between 10 and 21 h, with only one of these hearts lasting 24 h. The addition of intermittent left atrial (iLA) perfusion enabled real-time objective, quantifiable cardiac function assessment, a unique feature with a significant impact during the assessment of marginal and DCD donor hearts.

Overall, the underlying intention of this collection of articles was to emphasize the opportunity landscape for organ preservation techniques envisioned in Figure 1 and the betterment of perfusion outcomes, taking a closer look into cellular, molecular, and pathophysiological aspects of this continuously evolving area of research. The contributing papers provide extensive insight into clinical and engineering tools currently utilized to stimulate the development of better organ preservation techniques, supported by the most up-to-date research, device development, and clinical data. We hope that this issue serves as a foundation for further research in the areas that some of these articles have identified as lacking scientific information. These scientific gaps form the basis for identifying devices that can support prolonged graft perfusion and for moving from the current hurried logistical organization of transplant activities to a new logistics model involving organ repair centers where DBD and DCD organs can be repaired and possibly optimized during perfusion or optimized preservation. This new logistic may relaunch the organ transplant field, encountering the unmet clinical need of the many patients not receiving an organ or receiving an organ late when outcomes may be less enthusiastic.

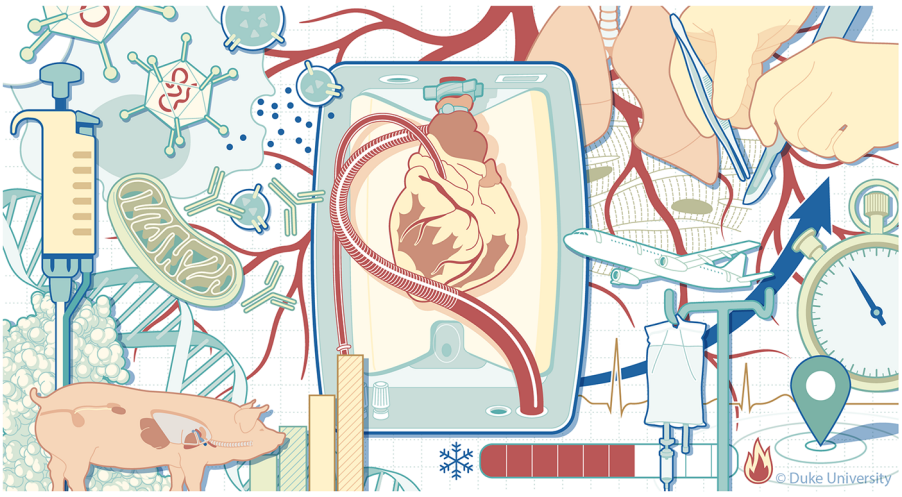

FIGURE 1
The challenges of extending machine perfusion length. Illustrated by Megan Llewellyn, MSMI (2024), copyright Duke University, licensed under CC BY-ND 4.0 with permission.

The availability of a new device that allows non-ischemic preservation of the heart through cold hypothermic blood perfusion, significantly reducing the risk of PGD (risk ratio 0.39) and adverse outcomes (5) promises to revolutionize the field further. If EVMP also expands the technologic armamentarium to pediatric donors and recipients, more patients will be transplanted whose needs are currently not encountered (6).

Author contributions

DB: Conceptualization, Writing – original draft, Writing – review & editing, Project administration, Supervision. JK: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. CA: Conceptualization, Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Video analysis of *ex vivo* beating hearts during preservation on the TransMedics® organ care system

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Background: Reliable biomarkers for assessing the viability of the donor hearts undergoing *ex vivo* perfusion remain elusive. A unique feature of normothermic *ex vivo* perfusion on the TransMedics® Organ Care System (OCS™) is that the donor heart is maintained in a beating state throughout the preservation period. We applied a video algorithm for an *in vivo* assessment of cardiac kinematics, video kinematic evaluation (Vi.Ki.E.), to the donor hearts undergoing *ex vivo* perfusion on the OCS™ to assess the feasibility of applying this algorithm in this setting.

Methods: Healthy donor porcine hearts ($n = 6$) were procured from Yucatan pigs and underwent 2 h of normothermic *ex vivo* perfusion on the OCS™ device. During the preservation period, serial high-resolution videos were captured at 30 frames per second. Using Vi.Ki.E., we assessed the force, energy, contractility, and trajectory parameters of each heart.

Results: There were no significant changes in any of the measured parameters of the heart on the OCS™ device over time as judged by linear regression analysis. Importantly, there were no significant changes in contractility during the duration of the preservation period (time 0–30 min, 918 ± 430 px/s; time 31–60 min, $1{,}386 \pm 603$ px/s; time 61–90 min, $1{,}299 \pm 617$ px/s; time 91–120 min, $1{,}535 \pm 728$ px/s). Similarly, there were no significant changes in the force, energy, or trajectory parameters. Post-transplantation echocardiograms demonstrated robust contractility of each allograft.

Conclusion: Vi.Ki.E. assessment of the donor hearts undergoing *ex vivo* perfusion is feasible on the TransMedics OCS™, and we observed that the donor hearts maintain steady kinematic measurements throughout the duration.

KEYWORDS

ex vivo perfusion, normothermic, video, kinematics, biomarker, cardiac transplantation

Introduction

Ex vivo machine perfusion has transformed organ transplantation outcomes by minimizing ischemic injury and reconditioning the organs prior to transplantation (1–3). Its use in clinical practice continues to grow as it has permitted for longer preservation times and for the utilization of the organs that would have traditionally been excluded from transplantation. In cardiac transplantation, normothermic *ex vivo* perfusion (NEVP) has allowed for the expansion of the donor pool through the utilization of hearts from donors after circulatory death (4). However, reliable measures for assessing the functionality and health quality of the donor heart remains elusive.

Currently, lactate measured in the perfusate is viewed by many as a proxy for injury and stress of the donor heart over time. Despite this, it has been well described that lactate is a poor predictor of post-operative graft outcomes (5, 6). A unique feature of NEVP is that the donor heart is maintained in a beating state throughout the preservation period and can be directly observed to assess the quality of the donor organ (7). Cardiac transplant surgeons can qualitatively assess the contractility of a donor heart as a parameter to determine its fitness for transplantation.

We applied a well-characterized video method for *in vivo* assessment of cardiac kinematics called video kinematic evaluation (Vi.Ki.E.) (8–10) and assessed the feasibility of using this method to measure *ex vivo* cardiac kinematics while a porcine donor heart is undergoing NEVP on the TransMedics® Organ Care System (OCS™). A successful measurement of cardiac kinematics while the donor hearts are beating on the OCS™ could allow for this technology to be applied as a biomarker to predict cardiac fitness.

Methods

Donor heart procurement and *ex vivo* perfusion

This study was approved by the Duke University Institutional Animal Care and Use Committee. Female Yucatan pigs (Sinclair Bio Resources, Auxvasse, MO, United States) aged 7–9 months were utilized for this study. Baseline cardiac troponin I values and cardiac magnetic resonance imaging (cMRI) were obtained prior to surgery. In preparation for surgery, the animals were anesthetized and intubated for mechanical ventilation. The donor hearts ($n = 6$) were procured in a standard fashion through a sternotomy. The hearts were then prepared on a back table and subsequently mounted on an OCS™ and underwent 2 h of NEVP at 34°C–35°C as described by Mendiola Pla et al. (11). During this time, the perfusion parameters of aortic flow, aortic pressure, heart rate, perfusion temperature, venous oxygen saturation (SvO_2), perfusate lactate, and perfusate hematocrit (Hct) were obtained.

Video acquisition

Video recordings were obtained using either a Nikon D5600 equipped with a Nikon 18–55 mm f/3.5–5.6G VR lens (Nikon Inc., Melville, NY, United States) or a Canon EOS Rebel T8i equipped with a Canon EFS 18–55 mm lens (Canon, Inc., Melville, NY, United States). Once perfusion of the donor heart was established on the OCS™ and the heart was beating, the camera was positioned approximately 30–40 cm perpendicularly in front of the heart (**Figure 1**). The distance between the camera and the heart, the focus, lighting, and orientation of the heart remained unchanged during and between recordings once these parameters were established. Serial high-resolution videos were recorded every 15 min during the perfusion period at a recording frequency of 30 frames per second (fps). All hearts were in normal sinus rhythm during the recordings.

Heterotopic heart transplantation and follow-up

Following the *ex vivo* perfusion and video acquisition, the hearts were cooled to 14°C–16°C, then arrested and removed from the OCS™. The heart was then prepared for transplantation in a standard fashion and transplanted into the recipient pig in an intra-abdominal position with the graft aorta anastomosed to the recipient aorta and the graft pulmonary artery anastomosed to the recipient inferior vena cava (11). Echocardiographic assessments of each transplanted heart were obtained between 2–6 post-operative days.

Quantitative analysis

Cardiac performance on the OCS™ was evaluated using Vi.Ki.E. by extraction of the kinematic parameters every 15 min during spontaneous beating, while monitoring the heart for 2 h (**Figure 1**). As shown in **Supplementary Video S1**, a virtual marker was placed on top of the beating heart and followed using a video spot tracker (VST), an open-software (https://cismm.web.unc.edu/resources/software-manuals/video-spot-tracker-manual/) capable of returning the XY coordinates of the marker movement for every cardiac beat. The selected VST kernel followed the heart movement and created the trajectory of contraction and relaxation (**Figure 2**) in the XY plane.

The coordinates are then analyzed using the Vi.Ki.E. system, which is written in MATLAB programming language (MathWorks, Inc., Natick, MA, United States) and returns the kinematic parameters such as contractility (maximal contraction velocity), cardiac force, energy expenditure, and trajectory perimeter (tissue compliance), as previously described by Rozzi et al. (10).

To investigate whether the kinematic parameters such as contractility (expressed as maximal contraction velocity), contraction force (indicating cardiac fatigue), energy (expenditure of energy during contraction/relaxation), and trajectory perimeter (indication of cardiac compliance) were modified over time, data acquisition was divided into four temporal windows (0–30, 31–60, 61–90, and 91–120 min) (**Figure 3**).

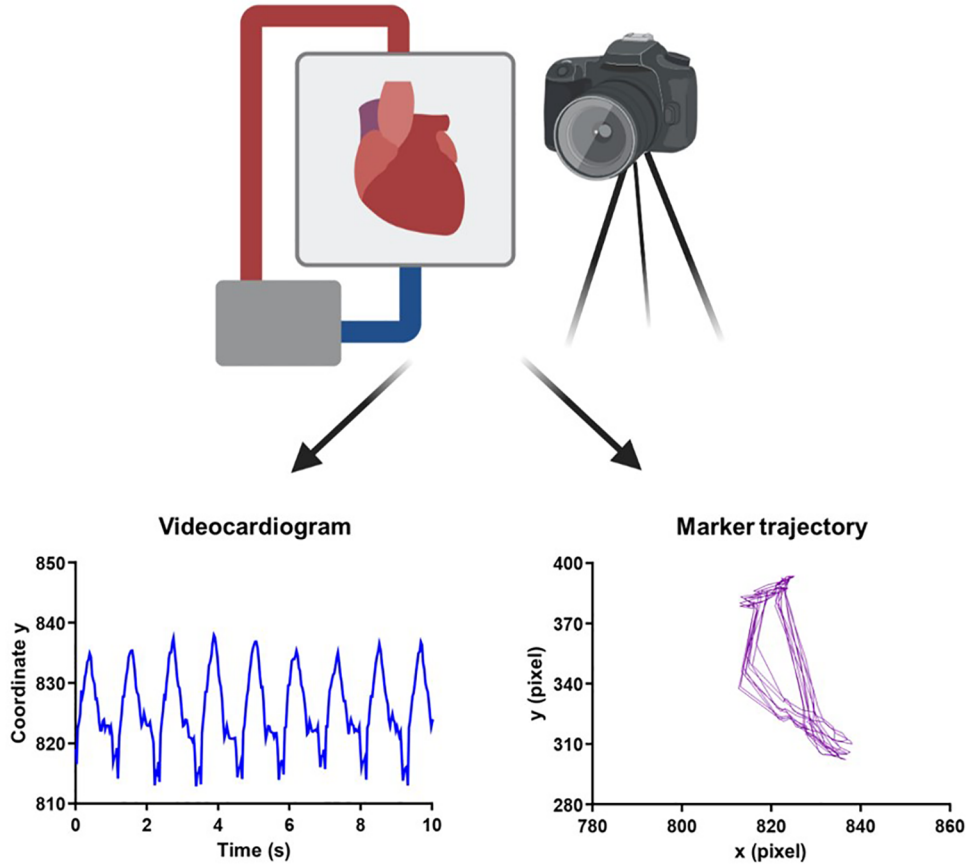

FIGURE 1
Experimental overview of video acquisition and kinematic analysis. Schematic representation of the camera positioned in front of the heart while it is undergoing normothermic *ex vivo* perfusion on the OCS. Using a Vi.Ki.E.-customized software, the trajectory of contraction (left to right) and relaxation (right to left) for every cardiac cycle was traced. A ViCG showing the displacement of a video marker with contraction/relaxation peaks and intervals among consecutive peaks was also traced. The schematic representation was created on BioRender. OCG, Organ Care System; Vi.Ki.E., video kinematic evaluation; ViCG, video cardiogram.

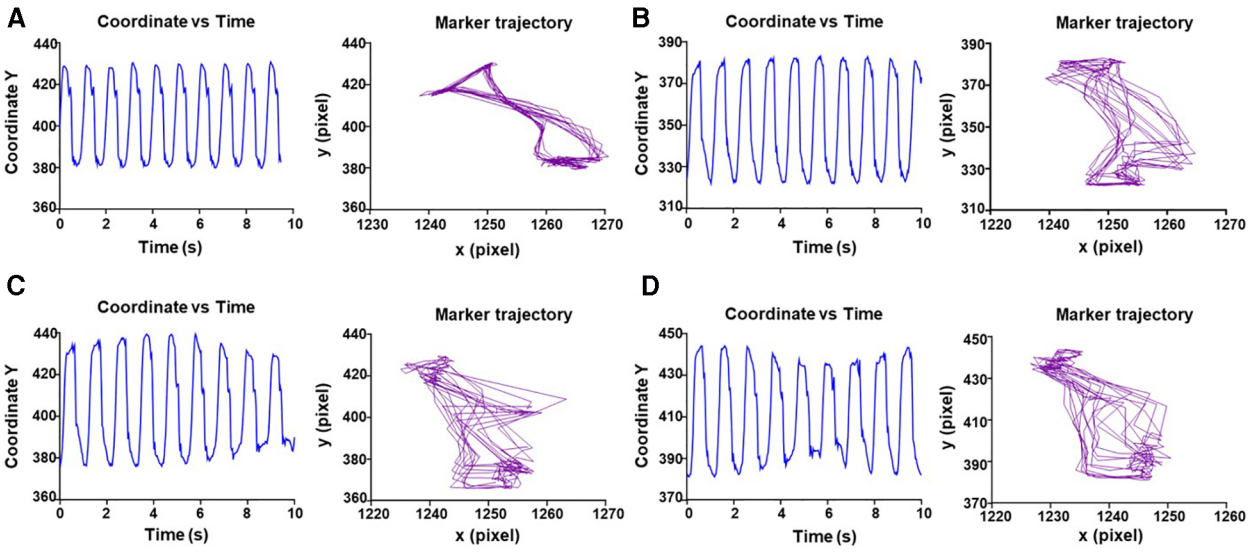

FIGURE 2
Representative evaluation of trajectory and displacement of marker 1 over time. (A) Displacement of video marker 1 with contraction/relaxation peaks and trajectory of contraction (left to right) and relaxation (right to left) for every cardiac cycle at 0 min. (B–D) Same as A for 30–60–105 min, respectively.

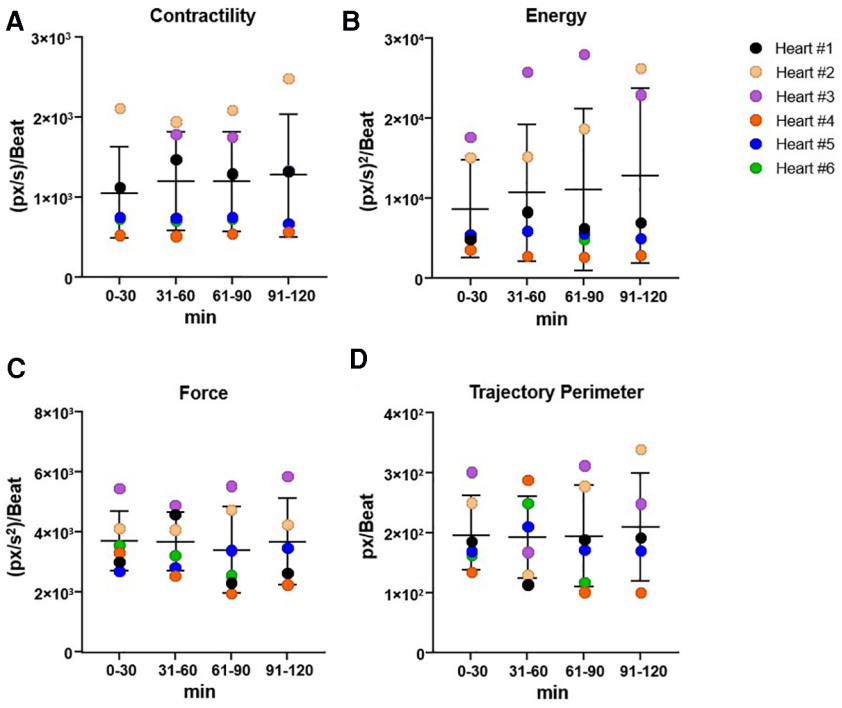

FIGURE 3
Overview of cardiac kinematic parameters over time. Cardiac kinematic parameters, such as cardiac fatigue (A), energy (B), contractility (C), and trajectory perimeter (D), grouped in range of 30 min, to evaluate the trend over time. The data used were obtained from a high-resolution video acquired every 15 min for 2 h, and analyzed with Vi.Ki.E. Data expressed as mean ± SD, using one-way ANOVA (significance set at $P < 0.05$). Vi.Ki.E., video kinematic evaluation.

Statistics

Data are expressed as mean ± SD. Normality was assessed by the Kolmogorov–Smirnov test. Comparisons were performed using one-way ANOVA with Bonferroni *post-hoc* test for multiple comparisons. Statistical analyses were performed using GraphPad Prism version 9.5.1 (GraphPad Software, San Diego, CA, United States). The details of the specific test used for each experiment are reported in the figure legends. *P*-values <0.05 were considered statistically significant.

Results

Pre-operative donor heart function and perfusion parameters

The baseline cardiac MRI demonstrated no evidence of compromised function in any of the donor hearts with the ejection fraction (EF) measuring >50%. The representative MRI data are shown in **Supplementary Figure S1** and **Supplementary Video S2**. The baseline troponin levels for each pig are shown in **Supplementary Table S1**. The median cardiac troponin I value was 22 ng/L with an interquartile range from 19 to 31 ng/L. Cardiac troponin I was elevated in only one of the pigs (865 ng/L) for unknown reasons. However, on gross inspection of the heart at the time of sternotomy, there was no evidence of cardiac injury or compromised activity.

The composition of OCS^{TM} perfusate is shown in **Supplementary Table S2**. It is donor blood-based and supplemented with several additives to maintain near physiologic function of the donor heart throughout the perfusion period. **Supplementary Figure S2** demonstrates the OCS^{TM} perfusion parameters measured throughout the perfusion period. The parameters were largely consistent between each of the donor hearts: average aortic flow, 0.61–0.72 L/min; average aortic pressure, 56.2–65.6 mmHg; average heart rate, 56–105 bpm; temperature, 33.9°C; SvO_2, 88.4%–96.0%; and Hct, 18.7%–28.4%. The average total perfusion time was 140 min with a standard deviation of 18 min. **Supplementary Figure S3** shows the plotted lactate trends of each heart during perfusion on the OCS, with minimal differences noted between each heart and each remaining within normal limits (<1.5 mmol/L).

Video kinematic parameters

While monitoring the heart for 2 h, we did not observe changes in spontaneous beating frequency (**Figure 2**). This may be attributed to the accommodation of the heart to the new environment. Despite the wide distribution of the data, likely due to differences between each of the hearts, we did not observe significant changes in both contractility and energy parameters over time in any of the hearts. This kinematic parameter ranged from 918.0 ± 430 px/s at the start of perfusion to 1,535 ± 728.5 px/s at the end of perfusion (**Figure 3A**). Force

measurements ranged from 3,776 ± 1,357 (px/s^2)/beat at the start of perfusion to 3,350 ± 897.4 (px/s^2)/beat at the end of perfusion (**Figure 3B**). Energy measurements ranged from 6,274 ± 3,240 (px/s)^2/beat at the start of perfusion to 16,948 ± 11,262 (px/s)^2/beat at the end of perfusion (**Figure 3C**). Finally, the trajectory perimeter measurements ranged from 199.3 ± 72.52 px/beat at the start of perfusion to 241.7 ± 83.59 px/beat at the end of perfusion (**Figure 3D**).

This was further assessed and confirmed by linear regression analysis (**Figure 4**). Contractility and energy showed a slightly increasing slope in the regression lines $y = 6.260 \times x + 969.8$ and $y = 91.49 \times x + 8{,}296$, respectively. On the other hand, force and trajectory perimeter exhibited a nearly flat regression over time in the regression lines $y = 2.937 \times x + 3{,}844$ and $y = 0.4509 \times x + 198.3$, suggesting that cardiac fatigue and tissue compliance remained constant over the period of the experiment.

Post-operative donor heart function

Each heart demonstrated robust biventricular contractility on post-operative echocardiography, which were all consistently performed by MMP. A representative recording is shown in **Supplementary Video S3**.

Discussion

We present the first report describing the utility of the Vi.Ki.E. system to assess the kinematics of *ex vivo* beating hearts undergoing normothermic perfusion on the TransMedics OCS^{TM}. This technology could potentially be utilized to provide quantitative assessments of cardiac fitness for the hearts preserved on the OCS^{TM} that could aid surgeons to decide whether a donor heart is suitable for transplantation. There are currently no reliable quantitative measures to assess donor heart fitness prior to transplantation. The utility of such a measure is important to be able to medically prepare for or even prevent outcomes of moderate or severe primary graft dysfunction (PGD). This is of great value since moderate PGD is associated with a 12% risk of mortality or re-transplantation and severe PGD with a 40%–50% risk (12). Lactate measured from the perfusate is the most used biomarker; however, it has been shown to correlate modestly with post-transplantation outcomes.

In this study, we chose to investigate four kinematic parameters that are essential for evaluating cardiac kinematic function: contractility, force, energy, and trajectory perimeter. Contractility refers to the maximal contraction velocity of the heart muscle, while force is an indication of cardiac fatigue. Energy represents the expenditure of energy during contraction and relaxation, and trajectory perimeter is an indicator of cardiac compliance. By monitoring these parameters over time, we aimed to determine if the performance of the heart changes during the *ex vivo* preservation time. To this end, we utilized healthy donor hearts to perform these studies. The results showed that there were no significant changes in any of the kinematic parameters over time. This suggests that the function and performance of the heart remain stable throughout the *ex vivo* preservation period on the TransMedics OCS^{TM}. Following the preservation period, we demonstrated that the cardiac allografts maintained robust contractility on post-transplantation echocardiography.

Given the ability to analyze cardiac fitness prior to transplantation in a non-invasive manner, future studies are warranted where Vi.Ki.E. is applied to analyze the kinematic parameters of human hearts undergoing NEVP on the OCS^{TM} and correlated with post-transplantation outcomes. The

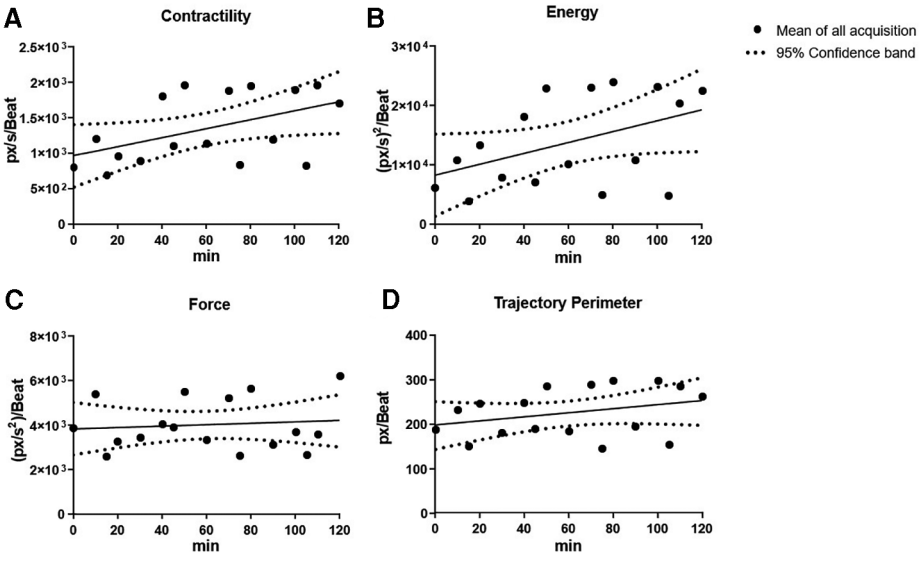

FIGURE 4
Overview of cardiac kinematics parameters over time with linear regression. Cardiac kinematic parameters [cardiac fatigue (A), energy (B), contractility (C), and trajectory perimeter (D)] were assessed over time using linear regression. Contractility and energy showed a slightly increasing slope, while force and trajectory perimeter had a nearly flat regression over time suggesting that cardiac fatigue and tissue compliance remained constant over time.

association of the kinematic measures with clinical outcomes could be used to develop an artificial intelligence (AI) platform that can predict PGD outcomes in patients based on the beating activity of the donor heart on the OCS™. The application of Vi.Ki.E. to guide AI assessments of the heart has been previously described (13). Potential translation of this technology to clinical practice would help to reduce subjective clinical decision making when assessing the donor hearts for transplantation and provide a possible standardized measure.

In conclusion, our study provides valuable insights into the performance of *ex vivo* beating hearts on the OCS™ system using the Vi.Ki.E. system. The results suggest that the cardiac function and performance remain stable on the OCS™, which is an encouraging finding for the expansion of the utility of normothermic *ex vivo* perfusion for donor heart preservation during transplantation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was reviewed and approved by the Duke University Institutional Animal Care and Use Committee.

Author contributions

DEB, MM, SB, MMP, GR, FM, and DCW performed the methodology, data acquisition and analysis, figures draft, and data interpretation. CAM and MMP performed the surgeries. AE performed perfusion of the hearts. FHL and RTG performed the video recordings. MB and LF performed software programming. FPlM performed statistical analysis. PL and MLB provided valuable advice and resources. MMP, SB, DEB, and MM drafted the final manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

PL was employed by TransMedics, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2023.1216917/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Representative baseline cardiac magnetic resonance imaging (MRI) of a donor heart. (A) Freeze frame of cine image during diastole (B) T1 mapping of cardiac MRI taken of donor heart prior to transplantation.

SUPPLEMENTARY FIGURE S2
Summary of measured OCS perfusion parameters.

SUPPLEMENTARY FIGURE S3
Plotted lactate trends measured from the OCS perfusate.

SUPPLEMENTARY VIDEOS1
Representative video of virtual marker tracing of a beating heart using Video Spot Tracker.

SUPPLEMENTARY VIDEO S2
Representative baseline MRI cine of donor heart before transplantation.

SUPPLEMENTARY VIDEO S3
Representative post-operative echocardiogram of allograft post-transplantation.

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Graft preservation in heart transplantation: current approaches

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Heart transplantation (HTx) represents the current best surgical treatment for patients affected by end-stage heart failure. However, with the improvement of medical and interventional therapies, the population of HTx candidates is increasingly old and at high-risk for mortality and complications. Moreover, the use of "extended donor criteria" to deal with the shortage of donors could increase the risk of worse outcomes after HTx. In this setting, the strategy of donor organ preservation could significantly affect HTx results. The most widely used technique for donor organ preservation is static cold storage in ice. New techniques that are clinically being used for donor heart preservation include static controlled hypothermia and machine perfusion (MP) systems. Controlled hypothermia allows for a monitored cold storage between 4°C and 8°C. This simple technique seems to better preserve the donor heart when compared to ice, probably avoiding tissue injury due to sub-zero °C temperatures. MP platforms are divided in normothermic and hypothermic, and continuously perfuse the donor heart, reducing ischemic time, a well-known independent risk factor for mortality after HTx. Also, normothermic MP permits to evaluate marginal donor grafts, and could represent a safe and effective technique to expand the available donor pool. However, despite the increasing number of donor hearts preserved with these new approaches, whether these techniques could be considered superior to traditional CS still represents a matter of debate. The aim of this review is to summarize and critically assess the available clinical data on donor heart preservation strategies employed for HTx.

KEYWORDS

heart transplantation, donor organ preservation, normothermic *ex situ* perfusion, hypothermic perfusion, graft preservation

1. Introduction

Heart transplantation (HTx) is the current gold standard surgical treatment for end-stage heart failure. However, despite improvement in the management of HTx recipients, the rate of primary graft dysfunction (PGD) continues to be relatively high, being a severe complication that still represents the leading cause of 30-day mortality after HTx (1–3). A recent national study from Sing et al. reported an overall incidence of PGD after HTx of 36%, with moderate-to-severe PGD rate of 32% (4). The interaction of donor, recipient and procedural variables has been shown to predispose to this life-threatening complication.

Continuous improvements of medical and interventional therapies, as well as the wide employment of mechanical circulatory support supports (MCS), have allowed an increasingly old population with multiple comorbidities to be considered as HTx candidates. On the other hand, the use of extended donor criteria to face graft shortage has increased the risk of worse outcomes after HTx. Therefore, in this setting, the strategy of donor organ preservation could play a central role in improving HTx recipient outcomes and in preventing PGD.

The use of static cold storage (SCS) for donor graft preservation, aims to stabilize biological tissues by influencing metabolic pathways. Such strategy slows the cellular and extracellular biochemical processes that are responsible for organ degradation during ischemic storage, thus extending a safe storage time up to several hours. On the other hand, machine perfusion (MP) systems permit to continuously perfuse the coronary arteries, reducing ischemic time and potentially mitigate the deleterious effects of ischemia/reperfusion injury. Despite the increasing number of donor hearts preserved with MP, whether MP could be considered superior to traditional CS still represents a matter of debate.

The aim of this paper is to summarize and critically assess the available clinical data on the donor heart preservation strategies currently employed for HTx.

2. Static cold storage

Employment of SCS aims to stabilize biological tissues by slowing the cellular and extracellular biochemical processes that are responsible for organ degradation during ischemic storage, thus extending the safe storage time. The temperature dependence of chemical reaction rates follows the "Arrhenius equation", used to describe the temperature-depending metabolic changes: for every 10°C reduction of temperature below the physiological temperature the metabolic rate for living biological tissues reduces by 50%. Hence, cold storage slows but does not completely arrest cellular metabolism. Consequently, progressive ischemic injury is an inevitable consequence of prolonged SCS and the results of HTx are suboptimal when graft ischemic time is greater than 6 h.

2.1. Cold solution and ice

The traditional ice-cold SCS remains the most commonly used technique for donor graft preservation, being both user friendly and cost-effective. In brief, after the donor heart is retrieved, it is placed into a sterile bag filled with 1,000 ml of preservation saline solution at 4°C which is then sealed into a second bag containing 1,000 ml of cold solution, and eventually in a third bag. Then, the heart is placed in a rigid sterile container filled with cold solution which is sealed and inserted into a cooler filled with ice for transportation.

Using conventional ice-cold SCS, prolonged ischemic time is known to be an independent risk factor for PGD and mortality after HTx (5–7). Moreover, the negative impact of graft ischemic time is considerably influenced by other donor characteristics, as age, left ventricular hypertrophy, mild-to-moderate coronary artery disease and catecholamine support (7).

As reported by the ISHLT Consensus Statement on donor heart and lung procurement (8), the ideal donor graft temperature during storage should probably be kept between 5°C and 10°C. In fact, freezing of any part of the heart is undesirable because freezing and subsequent thawing may cause tissue damage potentially responsible for PGD (9). Indeed, possible freeze injury was detected in 7% of autopsies done on patients deceased for clinically diagnosed PGD (1).

2.2. Controlled hypothermia with Paragonix SherpaPak cardiac transport system

The Paragonix SherpaPak™ cardiac transport system (PSP) is able to guarantee a constant, homogeneous and controlled temperature of the donor heart between 4°C and 8°C, thus minimizing tissue injury due to ice-cold temperature exposure.

The PSP device consists of two canisters, one internal and one external (**Figure 1A**). The internal canister is filled with cold storage saline solution (4°C–8°C), and the donor heart is submerged into it, after being connected to the canister lid by means of an aortic connector (**Figure 1B**). The most widely used solutions for heart preservation are the Celsior, the University of Wisconsin (UW) and the Custodiol (histidine-tryptophan-ketoglutarate—HTK). Then, the inner canister is placed into the outer one, creating an insulating air chamber, and outside this system is surrounded by single-use cooling ice packs. A thermometer connected with the internal canister allows continuous monitoring of the temperature (**Figure 1C**).

The GUARDIAN study is a post-market, observational registry of adult and pediatric patients who received a donor heart preserved and transported using either the PSP or standard preservation methods. Using data of 877 patents enrolled in the Guardian heart registry by 16 US centers, two cohorts of 249 patients were propensity matched according to the technique of graft preservation. Although the 1-year survival did not statistically differ between the two cohorts ($p = 0.12$), PSP preservation significantly reduced severe PGD rate, compared to ice-cold storage (4% vs. 10%, $p = 0.01$) (10). The use of PSP has also proved to be cost-beneficial. In a recent study that compared two groups of 87 matched patients (PSP and ice-cold storage), post-HTx costs were significantly lower when donor organs were preserved with PSP. This figure reflected a significant role of PSP in reducing incidences of severe PGD (5.7% vs. 16.1%, $p = 0.03$) and employment of mechanical circulatory support after HTx (21.8% vs. 40.2%, $p = 0.009$), and thus the recipient hospital length of stay (11).

Histological analyses of myocardial biopsies taken as soon as the donor hearts were reperfused during HTx, showed that grafts preserved with PSP appeared to have less interstitial edema and myocyte damage compared to those preserved with traditional ice storage (12).

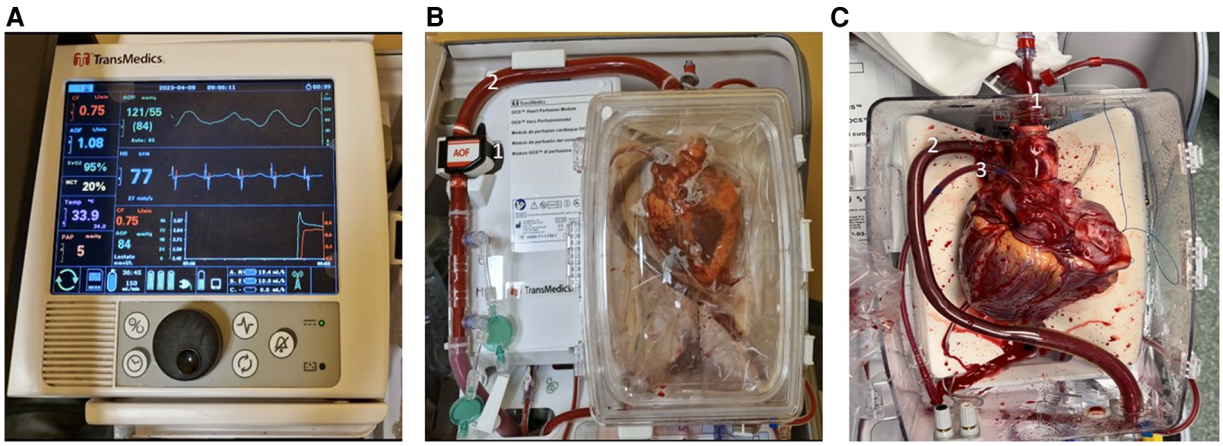

FIGURE 1
The transmedics organ care system. (A) Wireless monitor/controller. (B) Heart perfusion module: (1) aortic flow probe; (2) aortic perfusion line. (C) Instrumented heart: (1) aortic connector; (2) pulmonary artery cannula; (3) left ventricular venting tube.

3. Machine perfusion systems

Differently from static cold storage, MP systems represent dynamic methods of preservation that prevent extra ischemic time and thus potentially provide better preservation of the donor heart. Two types of MP systems are currently used in HTx: hypothermic (HMP) and normothermic (NMP) machine perfusion. As reported below in this paper, NMP could also provide the opportunity to assess the metabolic and functional status of the donor graft.

However, MP generally expensive and require well-trained and specialized personnel to set up the devices and manage any complications and malfunctions, which could otherwise damage the donor organ. Unfortunately, these limitations hamper a routine adoption of MP, and therefore their extensive employment is made even more difficult in centers with a low volume of activity and with scarce economic resources.

3.1. Hypothermic MP

The rationale of HMP preservation consists in reducing the metabolic requirements of the heart with an optimal and homogeneous cooling (below 10°C), while providing continuous metabolic support though perfusion with oxygenated, nutrient-enriched medium to limit as much as possible intracellular anerobic metabolism and consequent acidosis. Experimental studies performed on large animal models have suggested that compared to SCS, HMP could attenuate tissue injuries and provide superior myocardial function after HTx (13); nevertheless, clinical adoption of HMP has been limited due to the concerns about a reliable functional assessment of this system. Another major concern is related to the risk of edema during HMP preservation and after reperfusion. However, using a perfusate of high osmotic and/or oncotic power at low perfusion pressures could prevent edema formation. Moreover, edema related to HMP employment was reported to be more likely interstitial and reversible, with limited impact on post-HTx cardiac function (14).

Three single-center clinical studies have so far analyzed the effects of HMP using three different perfusion solutions: Wicomb et al. in 1984 used crystalloid cardioplegic solution in 4 patients (15), Hill et al. in 1997 used colloid cardioplegic solution in 8 patients (16), and more recently, in 2020, Nilsson et al. reported their experience employing a home-made MP with hyper-oncotic cardioplegic solution supplemented with hormones and erythrocytes, so called "non-ischemic hypothermic perfusion" (NIHP) (17). In their series, 6 patients who received donor hearts preserved with NIHP showed better outcomes 6 months after HTx compared to 25 recipients who received SCS preserved grafts (100% vs. 84% survival rate). Based on these preliminary promising results, the Xvivo Perfusion AB (Goteborg, Sweden) has patented the NIHP and further developed it to a commercially available device; currently, a randomized clinical trial is ongoing to assess patient and graft survival comparing NIHP to SCS (18).

The Lifecradle® Heart Preservation System is a HMP, currently under development, that uses hypothermic, oxygenated, nutrient perfusion at 5°C, in a controlled and monitored environment. The safety and efficacy of this device will be defined on the basis of clinical evidence, currently pending.

3.1.1. XVIVO perfusion

The XVIVO Heart Perfusion System consists of a roller pump, an oxygenator, a leukocyte filter and a cooler/heater. After cardiectomy, the donor heart is connected to the XVIVO device with an aortic cannula. Then, it is submerged into the reservoir, filled with 2.5 L of perfusion solution to which 500 ml of donor and recipient immunologically-compatible irradiated blood are added. The oxygenated perfusion solution (with a hematocrit of about 15%) is pumped into the aortic root to maintain the pressure of 20 mmHg to provide a coronary blood flow between

150 and 200 ml/min in a non-beating heart state. The temperature is constantly maintained at 8°C and the pH at 7.4 value. During transportation the XVIVO device does not need continuous monitoring and power source.

The initial Australian experience on 13 patients with the Xvivo NIHP for HTx showed promising results. In fact, there was no post-operative mortality and only 1 patient required veno-arterial extracorporeal membrane oxygenation (ECMO) due to secondary graft failure. The authors reported a median donor graft ischemic time of 404 min but since the period of non-ischemic perfusion was included this data could be misleading (19).

The XVIVO innovative technology was employed for xenograft preservation during the modified pig-to-human cardiac xenotransplantation performed at the Maryland University on January 2022 (20).

3.2. Normothermic MP

Normothermic MP systems perfuse the heart with oxygenated blood and enriched solutions, keeping it beating and at a near-physiological temperature of about 34°C. Currently the Organ Care System (OCS, TransMedics Inc, Andover, MA) represents the only NMP system commercially available for clinical use in HTx. *Ex vivo* perfusion with this device is particularly attractive when "extended criteria" for donor organs procurement have to be further evaluated; this system, besides limiting graft ischemic time, allows a real-time monitoring of the donor graft assessing hemodynamic parameters and lactate concentration, the latter being the main marker of organ metabolism, with a timely identification of potentially unsuitable grafts. Moreover, for these reasons, OCS is increasingly employed in resuscitation and assessment of organs from donation after circulatory death (DCD).

3.2.1. The organ care system

The OCS consists of a portable platform and is composed of a wireless monitor/controller (**Figure 2A**) and a circuit in which the donor blood perfuses the beating and empty donor heart (**Figure 2B**). The donor blood is mixed with a specific priming solution which contain mainly mannitol, electrolytes, vitamins and antibiotics; during *ex vivo* perfusion two other solutions are infused into the circuit: the catecholamine solution, containing epinephrine to replenish the depleted catecholamine level and the maintenance solution enriched with adenosine, aiming to modulate the coronary artery resistance.

The donor blood is oxygenated and maintained at 34°C by a heater-membrane oxygenator module, and it is delivered into the aortic inflow cannula by a peristaltic pump, after both venae cavae are closed. The blood perfuses the coronary vessels, reaches the coronary sinus and eventually the pulmonary artery, where an outflow cannula collects it closing the perfusion circuit (**Figure 2C**). The blood that does not reach the coronary sinus (because of aortic regurgitation and bleeding from cut surfaces), is collected and re-infused into the circuit. Flow and pressure of the blood are registered by probes. Stopcocks permit sampling of blood for arterial and venous lactate concentration monitoring. Coronary resistance, arterial pressure and coronary flow can be modified by acting on the pump flow speed and/or the maintenance solution infusion rate. OCS settings are adjusted to keep the mean aortic pressure between 80 and 100 mmHg, and coronary blood flow between 700 and 900 ml/min. Graft function is assessed by continuous monitoring of aortic pressure, coronary flow and the total arterial and differential artero-venous lactate profile. An arterial lactate level >5 mmol/L is considered an index of myocardial damage and thus a contraindication to use the graft, as well as an unfavorable artero-venous lactate production pattern as evidenced by a venous lactate concentration higher than arterial lactate level. The OCS device can be transported either by car, plane, or helicopter.

4. NMP employment

During the last decade, normothermic *ex vivo* perfusion has emerged as a key factor in expanding the cardiac donor pool, as it favors a safer employment of donor hearts selected using

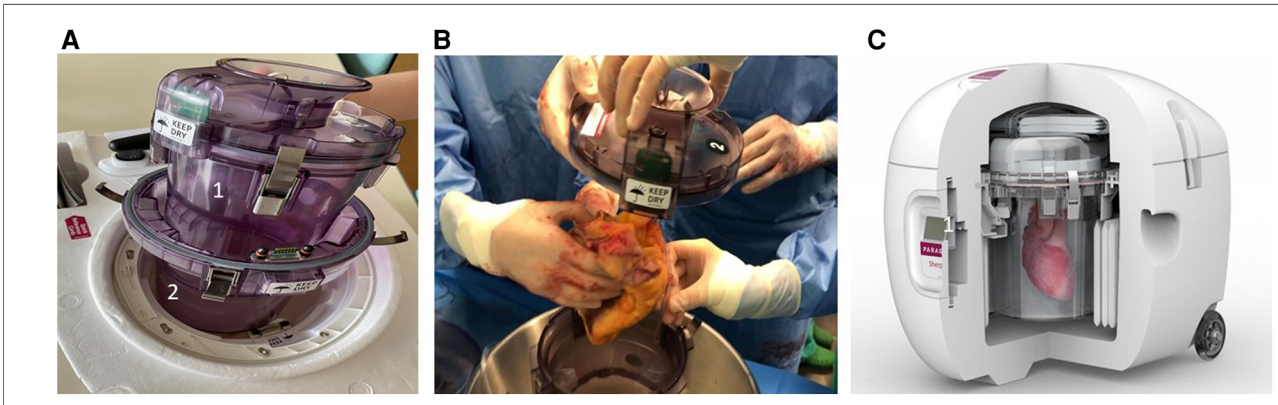

FIGURE 2
The paragonix sherpa-Pak system. (A) Internal (1) and external (2) canisters of Paragonix Sherpa-Pak. (B) The donor heart connected to the internal canister lid. (C) Overview of the system: (1) display and bluetooth data transmission module.

extended criteria by limiting ischemic time and allowing graft assessment. The following are some of the advantages of using NMP.

4.1. To shorten the ischemic time

The continuous coronary perfusion by means of oxygenated enriched blood is the main advantage of normothermic machine perfusion. The results of the PROCEED II controlled trial demonstrated the non-inferiority of the NMP compared to the traditional ICS. Although patient and graft survival between both study arms were similar, the OCS group reported a significantly shorter graft ischemic time. Interestingly, the 4 hearts of OCS group that were discarded because of an increasing lactate concentration, after histological analysis revealed signs of infarction, contusion and severe unrecognized left ventricular hypertrophy (21).

The OCS could be an effective tool to ensure graft quality when the expected graft ischemic time exceed the traditional "safe threshold" of 4 h, favoring long-distance organ retrieval. Two case reports presented successful HTx after preservation times of a donor heart as long as 10 h (22) and 16 h (23). The relatively safe non-ischemic "out of body time" could also be advantageous in particular situations, such as when an unexpected finding is discovered during organ retrieval that needs a histological definition (24).

In **Table 1** are reported graft ischemic and "out of body" time data derived from single-center and retrospective studies (25–31) when grafts were preserved with OCS.

4.2. To assess organ adequacy

NMP has shown interesting and promising results when utilized in extended-criteria DBD donors, and DCD. Extended-criteria were generally defined according to these parameters: >50 years of age, a history of drug abuse, cardiac resuscitation, coronary artery disease (CAD), expected graft ischemia time >4 h, left ventricular ejection fraction (LVEF) <50%, or interventricular septum thickness (IVS) >14 mm.

Data regarding marginal DBD donors are derived mainly from single-center observational studies. A previous report from our group demonstrated that NMP, compared to ICS in extended-criteria donor hearts, seemed to provide more stable hemodynamic conditions after HTx, reducing complications and allowing optimal outcomes. In fact, 5-year survival of OCS-preserved-heart group was 100% vs. 73% of CS control group ($p = 0.04$). These results are also supported by histopathological and ultrastructural evidence, suggesting better myocardial preservation in NMP grafts (28).

The EXPAND trial, which was designed as a single-arm multicenter study, evaluated the impact of OCS preservation in extended-criteria donor hearts. Out of 93 donor hearts evaluated, 75 were utilized for HTx (81% utilization rate). The 30-day post-HTx survival rate was 94.6% and the incidence of severe PGD in the first 24 h was 10.7%. Moderate to severe PGD was observed in 14.7% of patients (32).

In our experience, out of 74 grafts preserved with NMP, a total of 9 grafts were discarded (88% utilization rate) due to a progressive increase in lactate concentration, expression in most cases of severe left ventricle hypertrophy, scarring and undiagnosed coronary artery disease. In one case, NMP real-time evaluation of lactate trend permitted to discard an apparently adequate organ which, at gross pathological examination, revealed a dissection of the right coronary artery at 4 mm from its origin (33). Considering organ assessment and expansion of donor pool, interestingly at our center a donor heart with a myocardial bridge, which should be a relative contraindication to Htx, was successfully and safely transplanted in a 66-year-old recipient. This was possible owing to the continuous evaluation of cardiac function, which allowed to consider such graft suitable for HTx (34).

In case of DCD, the heart is exposed to prolonged periods of warm ischemia and to right atrial and ventricular over-distension during cardiocirculatory arrest, with possible irreversible myocardial injury. Thus, a post-asystolic functional assessment is of paramount importance when evaluating these hearts. In clinical practice, DCD hearts are retrieved with either direct procurement and perfusion (DPP) or normothermic regional perfusion (NRP). In DPP, the heart is removed after confirmation of death and expeditiously reperfused using the OCS (35–38). Instead, in NRP the employment of ECMO or cardiopulmonary bypass (CPB) facilitates cardiac resuscitation.

TABLE 1 Studies of normothermic machine perfusion for hearts from DBD with and without ICS as control group.

Author	OCS group					ICS group			
	no. of pts	Donor age (range)	Recipient age (range)	Out of body time (min)	Graft ischemic time (min)	no. of pts	Donor age (range)	Recipient age (range)	Graft ischemic time (min)
Ardehali et al. (21)	67	35 (18–58)	56 (20–75)	324 ± 79	113 ± 27	63	34 (13–60)	57 (20–76)	195 ± 65
Garcia Saez et al. (25)	26	37 ± 12	43 ± 13	371 ± 102	87 ± 15	–	–	–	–
Kaliyev et al. (26)	13	43 ± 15.5	40 ± 12	330.3	83 ± 8	–	–	–	–
Koerner et al. (27)	29	36 (17–54)	50 (37–64)	297	52	130	–	50.7 (37–64)	–
Sponga et al. (28)	14	46 ± 11	64 (35–75)	452	132 ± 28	24	44 ± 13	57 (30–73)	225 ± 48
Sponga et al. (29)	21	47 ± 11	58 (24–66)	272 ± 65	145 ± 29	79	48 ± 13	60 (28–73)	213 ± 63
Sato et al. (30)	16	–	52 ± 15.5	362 ± 153	114 ± 51	18	–	59 ± 16	183 ± 34
Rojas et al. (31)	68	37	49 ± 13	381 ± 74	115 ± 43	51	44.5	59 ± 13	228 ± 43

DBD, donation after brain death; OCS, organ care system; ICS, ice-cold storage.

After the donor is weaned from circulatory support, the heart is assessed "*in situ*" and if adequate recovery is observed it is retrieved and preserved with OCS or ICS (39, 40).

The introduction of NMP in clinical practice has permitted to utilize DCD donor hearts with gratifying results (35–40). Furthermore, when compared with the current "gold standard" ICS-preserved DBD hearts, the OCS-preserved DCD grafts have shown to provide comparable results (36, 40). A recent randomized controlled trial compared the outcomes of 90 HTx using DCD hearts reanimated, preserved and assessed with the OCS with that of 90 HTx perfomed by using DBD hearts preserved with ICS. The use of OCS resulted in a high rate of graft utilization rate (89%) in DCD group, and criteria for graft non-use were rising lactate concentrations, visual contractility anomalies or both. The 6 months risk-adjusted survival of HTx from DCD grafts (94%) was noninferior to that after transplantation of DBD hearts (90%). However, the rate of severe PDG was higher in HTx from DCD (15%) vs. DBD (5%) grafts (41).

The method of retrieval (DPP or NRP) was not associated with different outcomes after HTx according to the results reported in the experience of Messer et al. (40).

4.3. To facilitate HTx in high-risk patients

Heart MP could also play a protective role in high-risk recipients, particularly in those supported by durable mechanical circulatory support or who have undergone previous complex operations (25, 29, 31). HTx in these patients might be technically demanding and often requires a tedious dissection and prolonged CPB to complete the removal of intrathoracic ventricular assist devices or the isolation of the cardiac structures. The use of MP allows optimization of coordination between retrieval and implanting teams, favoring a meticulous and stress-free preparation of the recipients while the donor graft remains perfused. Moreover, this might reduce post-procedural bleeding and transfusions of blood products with improvement of hemodynamic stability after HTx. In a previous report from our group, in a series of patients bridged to HTx with MCS, OCS perfusion conferred a protective role regarding PGD development after HTx, compared to CS (7% vs. 42%, $p = 0.03$) (29).

4.4. To recover the injured graft

Sarcomere changes, such as Z-line thickening and/or non-orthodox banding were reported in donor hearts immediately after retrieval (28). After *in situ* reperfusion during HTx, hearts preserved with ICS are frequently affected by myocardial injury, with damage of contractile myofilaments and organelles, including mitochondria. The *ex situ* perfusion with OCS is reported to be effective in reconditioning donor hearts, that are maintained metabolically active and able to heal ultrastructure changes (28, 42).

Donor hearts, selected according to expanded-criteria, appear to be best treated by NMP, especially when severe hypotension or cardiac arrest occurs during the retrieval phase and since this technique could hamper the negative effect of cold storage on ultracellular cardiac function (42).

5. Future perspectives

MP systems could be useful platforms for cardiac conditioning before transplantation, since they create a "safe period" between procurement and transplantation during which the organ could potentially be manipulated. Graft immunomodulation, via infusion of viral vectors (43, 44) or mesenchymal stem cells (MSC) injection (45), could modify its immunogenic capacity and reactivity.

In HTx, donor infusion of MSCs has been shown to prolong the survival of a semi-allogeneic HTx in a mouse model through the generation of regulatory T cells (46). In addition to MSC, also the injection of extracellular vesicles secreted from cardiomyocytes (iCM-EVs) derived from induced pluripotent stem cells have been demonstrated to lead to functional recovery hearts injured from pathologic hypertrophy. Since their content is mainly composed of miRNAs that modulate specific cardiac processes, they could represent a promising cell free alternative for cardiac recovery (47). MP could also represent the ideal platform for the introduction of viable and competent mitochondria into the graft tissue prior to reperfusion to improve the heart metabolic function and to reduce the ischemia-reperfusion injury (48). Preliminary scientific reports, confirming the potential for clinical application of these techniques, underline the need for prolonged graft manipulation in order to achieve a significant effect, making NMP an irreplaceable method (43–49).

In an effort to further suppress tissue metabolism and thus increase a safe preservation duration, sub-zero preservation techniques have been investigated in preclinical studies (50). Isochoric supercooling, that limits the cristallization of ice by controlling temperature and volume systems, and vitrification, that involves a large amount of cryopreservation and a rapid cooling scheme, are two intriguing techniques for "freezing biological time". Despite experimentary results on cells, tissues and small-animal organs are encouraging, successful employment to larger-volume organs remains to be demonstrated (51).

Various pharmacological agents have also been investigated in order to better preserve the graft by interfere with the ischemia-reperfusion injury mechanism. Donor simvastatin treatment might significantly improve graft function after transplantation (52), while valproic acid seems to stimulate cardioprotective immune-metabolomic pathways (53).

Interfering with the immune system during graft preservation could lead to a smooth immunological response of the recipient against the organ, and thus reduce the degree and number of rejections. Antioxidative agents, and inhibitors of cytokines production and activity and maturation of lymphocytes, as well as inhibitors and modulators of cellular receptors involved in signalling pathways and adhesion molecules expression are described as intriguing potential treatments (54).

TABLE 2 Characteristics of current preservation techniques for cardiac grafts.

Technique	Cost	Temperature control	Reduction of IT	Function assessment	Expertise/complexity	Graft manipulation
ICS	+	+	–	–	+	–
PSP	++	+++	–	–	+	–
OCS	+++	+++	+++	++	+++	+
NIHP	+++	+++	++	–	++	NA

IT, ischemic time; ICS, ice cold storage; PSP, Paragonix SherpaPak; OCS, organ care system; NIHP, non-ischemic hypothermic perfusion; NA, not available.

6. Conclusions

The field of graft preservation is subject of notable innovations. ICS was the standard technique for 50 years, but it does not allow temperature monitoring and exposes the heart to freezing damage. Increasingly, the use of ICS is being replaced with controlled hypothermic preservation using the Paragonix SherpaPak™ device, that in preliminary reports seems to offer advantages over ICS in therms of better organ preservation and clinical outcomes. Paragonix SherpaPak™ transport system has all the premises to be considered the near-future standard for donor heart cold storage, being able to allow temperature control, avoid tissue freezing, be relatively cheap and simple to use.

On the other hand, MP systems can represent the opportunity to assess and recondition the donor heart and are increasingly employed worldwide in an attempt to expand the donor pool for HTx. However, despite interesting results, the role of MP in HTx remains still debatable, mainly because of higher costs and training needs than those required for CS.

At present, while the clinical effectiveness of HMP has to be investigated, NMP with the OCS seems to allow safe utilization of DCD and extended-criteria donor organs, combining two major advantages: to limit the graft ischemic time and to verify cardiac function by direct visual inspection and through assessment of metabolic values, and haemodynamic parameters. Maintenance of myocardial aerobic metabolism during preservation could lead to better donor heart quality compared to traditional CS. Thus, the NMP represents an effective technique that permits to expand the donor pool, allowing acceptance of grafts which would have otherwise been refused, while maintaining satisfactory safety levels. In fact, NMP allows to identify unsuitable grafts and discard them before transplantation, reducing the risk of PGD and its life-threatening sequelae (20, 27, 31).

Some issues related to MP systems should be more thoroughly investigated in the near future to further improve this technique, such as additional metabolic support, solution components and optimal perfusion settings. Also, the identification of other functional parameters or biomarkers, apart from lactate levels, could be of paramount importance to increase the sensitivity of MP to help clinicians in assessing suitability of perfused donor grafts.

The main drawback of NMP is that it requires an experienced and well-trained professional team, to manage the interaction between the donor organ and the *ex vivo* perfusion, and to promptly intervene in case of machine malfunction or user error. In fact, since the donor heart is preserved in a beating normothermic state, the margin of safety is limited in case of complications or non-appropriate NMP management due to the risk of catastrophic and irreversible warm ischemia of the graft (Table 2).

In conclusion, the satisfactory results reported in HTx with high-risk recipients and extended-criteria donors highlight the effectiveness of NMP in complex cases, particularly in unfavorable combinations of donor, procedural and recipient characteristics. The results of ongoing multicenter clinical trials investigating on heart MP are required to confirm these expectations.

Author contributions

AL: Conceptualization, Investigation, Writing–original draft, Writing–review and editing. SS: Supervision, Validation, Visualization, Writing–review and editing. GB: Validation, Visualization, Writing–original draft. AS: Data curation, Visualization, Writing–original draft. GG: Data curation, Investigation, Visualization, Writing–review and editing. CD: Visualization, Writing–review and editing. MM: Visualization, Writing–review and editing. CN: Writing–review and editing. DP: Writing–original draft. MB: Visualization, Writing–original draft. UL: Supervision, Writing–review and editing. IV: Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Case report: Heart retransplant from a donor after circulatory death and extended transport period with normothermic perfusion

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A 55-year-old man with end-stage heart failure, who had an orthotopic heart transplant 21 years prior, underwent heart retransplantation using a heart from a donor with circulatory death in a distant location and an extended transport period with normothermic *ex vivo* perfusion. Owing to the persistent and worsening shortage of donor hearts, this case illustrates that expanding the donor acceptance criteria to include more distant donor locations and enrolling recipients with extended criteria (e.g., heart retransplantation) is feasible.

KEYWORDS

heart retransplantation (RTx), normothermic *ex vivo* perfusion, donor distance, donor after circulatory death, organ preservation

Introduction

Orthotopic heart transplantation remains the gold standard of treatment for end-stage heart failure. Recently, heart transplantation from donors after circulatory death (DCD) has expanded, which was enabled by the usage of the FDA-approved transportable Organ Care System™ (OCS) (TransMedics, Andover, MA, USA) (1). This innovative device preserves the standard and extended-criteria for *ex vivo* donor hearts during normothermic *ex vivo* perfusion. Improvements in preservation and transportation conditions can improve organ quality at the time of transplantation, shorten the acceptable maximum allograft ischemic time, and optimize patient outcomes.

We describe our successful experience with a normothermic *ex vivo* perfusion system, using a heart from a DCD, for an extended transport period >7 h using our modified strategy. To our knowledge, this is the first report of heart retransplantation using a normothermic *ex vivo* perfusion system in DCDs.

Case description

A 55-year-old man with heart failure and reduced ejection fraction (EF 30%) following a heart transplant in 2001 was listed for heart retransplantation (2). Despite maximal medical therapy, the patient's condition deteriorated, requiring an implantable cardioverter and multiple hospitalizations. The patient was considered a candidate for heart retransplantation. A 24-year-old male with a compatible blood type was identified as a

suitable donor; the donor's heart had an acceptable size and sex match with the recipient (predicted heart mass, 1.36; height, 110%; body weight, 126%) and normal biventricular function. The heart was retrieved after circulatory death of the donor in a hospital located 700 miles away.

Organ donation and the subsequent withdrawal of life support were performed in the intensive care unit, which was separate from the thoracic and abdominal organ retrieval teams. Heparin was administered to the donor 5 min before withdrawal. The donor was observed after the cessation of circulation for 2 min, declared deceased, and quickly transferred to the operating room.

Median sternotomy and laparotomy were performed simultaneously with a venous cannula placed directly into the grossly distended right atrium to enable rapid collection of 1.2 L of blood to prime the *ex vivo* perfusion apparatus.

Heparin was added to the blood collection bags. An aortic cross-clamp was applied on the ascending aorta, and 1 L of cold del Nido cardioplegia solution was delivered via the aortic root. The heart was vented by cutting across the left lower pulmonary vein and the inferior vena cava at the pericardial reflection (3).

After cardioplegia was delivered, the heart was explanted with transection at the mid-aortic arch, distal to the main pulmonary artery bilaterally, across the superior vena cava at its confluence with the innominate vein.

Ex vivo preservation

The donor heart was attached to the Organ Care System (OCS^{TM}) after cannulation of the aorta and pulmonary arteries. according to the manufacturer's instructions (4), the Organ Care System circuit prime was made up by mixing 1.2 L of donor blood that had been passed through a leucocyte filter (Pall LeukoGuard BC2; Pall Corporation, Port Washington, NY, USA) with 500 ml of TransMedics Priming Solution containing buffered electrolytes and mannitol. Multi-vitamins, antibiotics, albumin and steroids were added to the system. A TransMedics proprietary maintenance solution (1 L) containing isotonic electrolytes, amino acids, dextrose-insulin, and low-dose adenosine was infused at a rate of 0–30 ml/h during *ex vivo* perfusion to maintain the coronary flow within an acceptable range of 650–900 ml/min. The heart started beating spontaneously in a sinus rhythm and did not require pacing. A vent was placed via the left atrium to decompress the left ventricle. There was no PFO or atrial septal defect found in this donor. The right atrial appendage incision which was made previously for donor blood collection, superior and inferior vena cavae were securely closed. The heart was positioned such that oxygenated blood directly entered the ascending aorta, flowed down the coronary arteries, returned to the right side of the heart, and diverted into the pulmonary artery before draining into the circuit reservoir. This apparatus principally uses aortic pressure, coronary flow, and arteriovenous lactate concentrations to assess cardiac function; a lower venous concentration indicates lactate uptake and satisfactory myocardial function. An infusion of low-dose adenosine, another infusion containing adrenaline, and adjustable circuit pump flow were used to control coronary vascular resistance and heart rate to keep parameters within the following ranges: aortic pressure 65–90 mmHg, coronary flow 650–900 ml/min, and heart rate 65–100 beats per min. The coronary inflow and effluent ports on the perfusion circuit were simultaneously sampled at regular intervals to measure myocardial lactate extraction. Lactate concentrations in the perfusate were measured using an automated iSTAT analyzer (Abbott, Princeton, NJ, USA).

We began the transplantation when the perfusion and lactate profiles met the OCS parameters. Once stable OCS pump flow, several initial downward trends in serum lactate concentrations, and biventricular motion were confirmed, we administered general anesthesia and placed arterial and venous monitoring lines. The difference in arteriovenous lactate levels improved and remained stable at less than 5 mmol/L. While the heart was being transported, the recipient underwent a repeat median sternotomy. Following successful sternal reentry, extensive dissection was performed, confirming hemostasis.

As soon as the OCS arrived in the operating room, the transportable *ex vivo* perfusion was turned off, supplemented cold del Nido cardioplegia solution was delivered to the donor heart with prompt electromechanical arrest, and the heart was removed from the OCS for implantation. This reduced the total *ex vivo* heart perfusion time.

Cardiopulmonary bypass was initiated at 34°C with aortic and bicaval cannulations, and cardiectomy was performed. During this process, the heart was cooled on ice for 30 min, which was expected to reduce oxygen demand and afford adequate cellular protection (5).

Implantation

Donor heart implantation was performed with left atrial anastomosis, followed by ascending aortic anastomosis. During heart reperfusion, the remaining cardiac anastomoses, such as the pulmonary artery, inferior vena cava, and superior vena cava, were performed using an end-to-end anastomosis technique. This modified implantation technique (6) shortened the second warm ischemic time, reduced the aortic cross-clamp time, and secured an additional reperfusion period for the implanted heart. Although no electrical activity or ventricular squeezing was found in the initial 150 min of the reperfusion period, atrioventricular conduction and normal sinus rhythm were promptly regained. At the time of separation from cardiopulmonary bypass, an inotropic agent was administered to maintain a cardiac index of 2.5 L/min/m^2.

The total *ex vivo* heart perfusion time was 423 min. The allograft ischemic time was 107 min, including the first and second warm ischemic times of 9 min and 15 min, respectively. The recipient cardiopulmonary bypass and aortic cross-clamp times were 233 min and 50 min, respectively. The patient recovered well and was discharged on postoperative day 17. Six months after transplantation, the patient continued to have excellent graft function without any evidence of rejection.

Discussion

This report describes a successful clinical heart retransplantation using a heart from a DCD with an extended transport period and an *ex vivo* cardiac perfusion device.

The use of organs from DCDs has been successful for heart transplantation, which has helped reduce the discrepancy between the number of patients awaiting transplantation and the number of suitable donors. Strong endorsements for such transplants by national and international regulatory bodies have led to the wider adoption of this strategy, with organs from DCDs contributing to an increasing percentage of the total number of donors worldwide. Donor selection in DCD donations is the same as donation after brain death (DBD) scenarios, avoiding size mismatch based on predicted heart mass ratio. According to OCS heart EXPAND trial, marginal donors with an anticipated total ischemic time more than 4 h or age >50 years are now included. Further cardiac evaluations, echocardiography and cardiac catheterization, are required prior to withdrawal of life support (7).

Our retrospective outcomes after 50 years of experience of heart retransplantation demonstrated inferior short-term survival compared to primary transplantation. The decision of heart retransplantation listing is made by our multidisciplinary team based on case-by-case basis and we do not reluctant to offer heart retransplantation for candidates with severe graft dysfunction and have no other options. Given the inferior outcomes, careful candidate selection is recommended to optimize donor heart utilization (2).

Following DCD transplant, the incident of severe primary graft dysfunction (PGD) is found to be higher compared to similar DBD recipients. The pathophysiology of PGD is not well addressed in DCD hearts but thought to be due to functional warm ischemia occurring during and post withdrawal of life support. The study showed DCD recipients with severe PGD required shorter duration of mechanical circulatory supports (MCS) and spent fewer days in ICU and hospital compared to similar DBD recipients suggests DCD heart procurement process may contribute to a period of delayed graft function with subsequent rapid recovery which may differ from what we observed in DBD recipients with severe PGD (8). Therefore, our institution has low threshold of using MCS postoperatively in extended-criteria donor heart recipients. We considered using peripheral veno-arterial extracorporeal membrane oxygenation (VA-ECMO) as the main strategy to support PGD patients not only it is less invasive and promptly available but also promote lower rate of mediastinal infection. Intra-aortic balloon pump (IABP) support is routinely combined with VA-ECMO for the treatment of severe PGD requiring ECMO therapy for the benefit of augmentation of coronary perfusion, provide peripheral pulsatility and promote afterload reduction. MCS will be discontinued as soon as heart function returns to normal to prevent further complications that increase with time (9).

Several cardiac perfusion devices are currently commercially available and more are being trialed. Commercially available portable *ex vivo* heart preservation systems have made it possible to maintain physiological perfusion of a donor organ coming from a distance. The device has been used for both the resuscitation and assessment of marginal hearts from DCDs for transplantation. Approximately 20% more transplantations could be done if hearts were donated after circulatory deaths (4).

We proposed a modified strategy, as shown in **Figure 1**, aiming to reduce the normothermic *ex vivo* perfusion period, provide 30–45 min of the organ-stabilizing period before implantation, reduce the second warm ischemic time, and secure additional

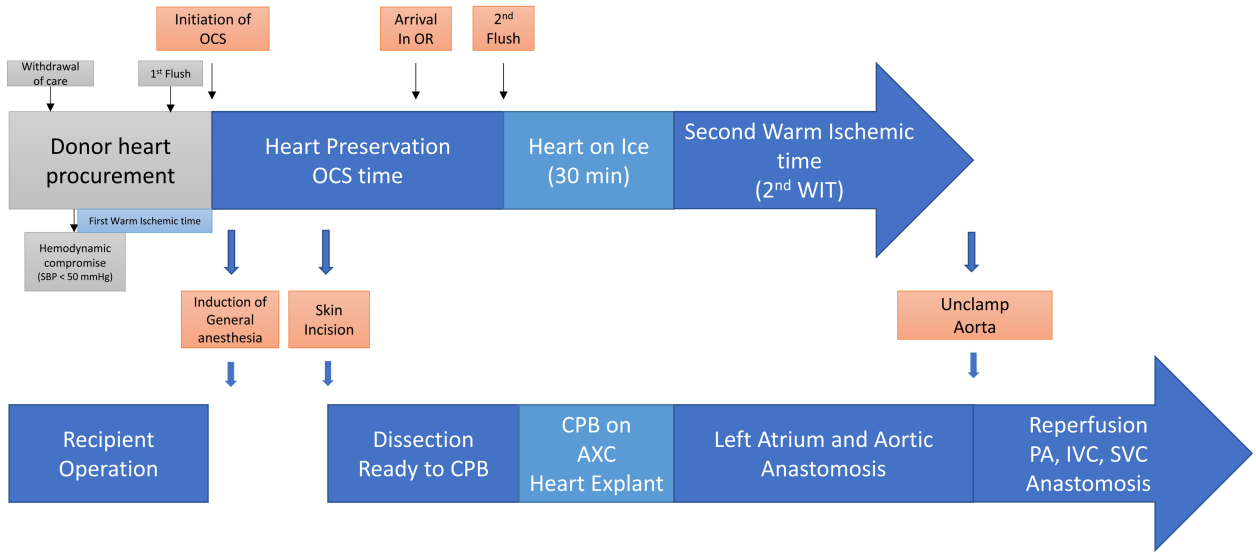

FIGURE 1
Transplanting a heart from a donor after circulatory death and extended transport period with normothermic *ex vivo* perfusion. The modified strategy was proposed to reduce normothermic *ex vivo* perfusion period, provide 30–45 min of organ stabilizing period before implanting, and reduce the second warm ischemic time, as well as secure the additional reperfusion time. It is anticipated to reduce the cardiopulmonary bypass time and aortic cross clamp time by optimizing the timing of the initiation of cardiopulmonary bypass and aortic cross clamp together with the timing of turning off *ex vivo* perfusion devices.

reperfusion time (10). This method can reduce the cardiopulmonary bypass and aortic cross-clamp times by optimizing the timing of the initiation of cardiopulmonary bypass and aortic cross-clamp, together with the timing of turning off the *ex vivo* perfusion device (10). We anticipate that the overall outcomes will improve transplantation methods with reduced cardiopulmonary bypass and aortic cross-clamp times.

Owing to the shortage of donor hearts, this case illustrates that expanding the donor acceptance criteria to include more distant donor locations and enrolling recipients with extended criteria is feasible. The expansion of heart transplantation from DCDs would maximize transplantation opportunities and reduce the time spent on transplantation waiting lists. Considering the increasing number of patients with end-stage heart failure awaiting cardiac transplantation, we believe that a regulated normothermic *ex vivo* perfusion device for DCDs is a useful strategy for maximizing organ allocation in select recipients.

Author's note

This subject was enrolled in the OCS Heart Perfusion Post-Approval Registry (NCT 05047068). The OCS Heart system is FDA approved for commercial use and patients will be followed per transplant center's standard of care protocols. A waiver of consent has been granted for the data collection by WCG IRB.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YS contributed to the design of the research and took the lead in writing the manuscript; PK, CR, and TK helped determine data metrics and collected the data; All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Composition of *ex vivo* perfusion solutions and kinetics define differential cytokine/chemokine secretion in a porcine cardiac arrest model of lung preservation

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Background: *Ex vivo* lung perfusion (EVLP) uses continuous normothermic perfusion to reduce ischemic damage and to improve post-transplant outcomes, specifically for marginal donor lungs after the donation after circulatory death. Despite major efforts, the optimal perfusion protocol and the composition of the perfusate in clinical lung transplantation have not been identified. Our study aims to compare the concentration levels of cytokine/chemokine in different perfusion solutions during EVLP, after 1 and 9 h of cold static preservation (CSP) in a porcine cardiac arrest model, and to correlate inflammatory parameters to oxygenation capacities.

Methods: Following cardiac arrest, the lungs were harvested and were categorized into two groups: immediate (I-EVLP) and delayed EVLP (D-EVLP), after 1 and 9 h of CSP, respectively. The D-EVLP lungs were perfused with either Steen or modified Custodiol-N solution containing only dextran (CD) or dextran and albumin (CDA). The cytokine/chemokine levels were analyzed at baseline (0 h) and after 1 and 4 h of EVLP using Luminex-based multiplex assays.

Results: Within 4 h of EVLP, the concentration levels of TNF-α, IL-6, CXCL8, IFN-γ, IL-1α, and IL-1β increased significantly ($P < 0.05$) in all experimental groups. The CD solution contained lower concentration levels of TNF-α, IL-6, CXCL8, IFN-γ, IL-2, IL-12, IL-10, IL-4, IL-1RA, and IL-18 ($P < 0.05$) compared with those of the Steen solution. The concentration levels of all experimental groups have correlated negatively with the oxygenation capacity values ($P < 0.05$). Protein concentration levels did not reach statistical significance for I-EVLP vs. D-EVLP and CD vs. CDA solutions.

Abbreviations

CSP, cold static preservation; DBD, donation after brain death; DCD, donation after circulatory death; D-EVLP, delayed *ex vivo* lung perfusion with Steen solution; D-EVLP/CD, delayed *ex vivo* lung perfusion with Custodiol-N solution supplied with dextran; D-EVLP/CDA, delayed *ex vivo* lung perfusion with Custodiol-N solution supplied with dextran + albumin; EVLP, *ex vivo* lung perfusion; I-EVLP, immediate *ex vivo* lung perfusion; IRI, ischemia–reperfusion injury; LTX, lung transplantation; PCA, principal component analyses; UHC, unsupervised hierarchical cluster analyses.

Conclusion: In a porcine cardiac arrest model, a longer period of CSP prior to EVLP did not result in an enhanced protein secretion into perfusates. The CD solution reduced the cytokine/chemokine secretion most probably by iron chelators and/or by the protecting effects of dextran. Supplementing with albumin did not further reduce the cytokine/chemokine secretion into perfusates. These findings may help in optimizing the preservation procedure of the lungs, thereby increasing the donor pool of organs.

KEYWORDS

graft preservation, *ex vivo* lung perfusion, porcine, Steen solution, Custodiol-N, dextran, albumin, cytokines/chemokines

1. Introduction

Lung transplantation (LTX) is the ultimate treatment option for patients suffering from end-stage lung disease. However, there is a huge discrepancy between available donor organs and patients with demand, resulting in high waiting list mortality (1). To overcome this disparity, transplantation centers have extended their selection criteria (2–4), and few countries have legalized the donation after circulatory death (DCD), aside from the donation after brain death (DBD). *Ex vivo* lung perfusion (EVLP) has been discussed as an alternative preservation technique to improve the outcome of marginal or DCD organs. In contrast to the standard procedure, which is based on cold static preservation (CSP) and thus reduced metabolism, the donor organ is perfused with normothermic solution during EVLP (5). Therefore, EVLP reduces the cold ischemic time and the ischemia–reperfusion injury (IRI) of the organ (6). Furthermore, EVLP allows to evaluate the condition of the graft and to therapeutically treat donor organs prior to implantation (7, 8). In experimental studies and human clinical trials, EVLP has shown promising results (9–11). Still, it is not clear whether the donor organ needs to be perfused directly after procurement or if a prolonged CSP prior to perfusion is harmful to the lungs. Moreover, the optimal composition of the perfusion solution in reducing IRI has not been defined.

This study aimed to examine whether immediate EVLP (I-EVLP), after only 1 h of CSP, or delayed EVLP (D-EVLP), after 9 h of CSP, would result in different cytokine/chemokine concentrations in perfusates. We used a porcine model as an established alternative to human LTX, in which we previously demonstrated a comparable lung function of immediate vs. delayed perfusion (12). Moreover, a Custodiol-N solution supplied with dextran was shown to have a positive impact on the functional parameters of the EVLP-preserved porcine lungs (13). In the present study, we investigated the impact of modified Custodiol-N solution on cytokine/chemokine patterns in perfusates and correlated the data to oxygenation capacity measurements collected from the same lungs (12, 13). The differences in cytokine/chemokine concentrations could help identify the ideal start point of perfusion and the optimal composition of the perfusion solution to preserve the DCD lungs with reduced IRI, resulting in improved transplant engraftment, survival, and ultimately increased donor pool.

2. Material and methods

2.1. Animals

All animals included in this study received human care compliant with the "Principles of Laboratory and Animal Care" and the Guide for the Care and Use of Laboratory Animals, which was composed by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NH publication no. 86–23, revised 1996). The study only involves organ procurement from animals because none of them underwent medical treatment prior to euthanasia. According to the applicable German law (§1 VTMVO), the study was reported to the local Landesamt für Natur, Umwelt und Verbraucherschutz NRW and supervised by the Central Animal Laboratory of the University Duisburg-Essen.

2.2. Chemicals

The compositions of the different perfusate solutions are displayed in **Table 1**. Steen Solution™ and Perfadex™ were purchased from XVIVO Perfusion (Gothenburg, Sweden). Custodiol-N was acquired from Dr. F. Köhler Chemie (Bensheim, Germany). Pyrogen-free dextran 40 (AppliChem, Darmstadt, Germany) was added to the modified Custodiol-N solution in a concentration of 50 g/L. The D-EVLP with Custodiol-N solution supplied with dextran + albumin (D-EVLP/CDA) solution was additionally supplemented with 7 g/L of bovine serum albumin (Carl Roth, Karlsruhe, Germany). The sterilization process of the solution was performed by filtration using a 0.22 µm filter (Filtropur BT25, Sarstedt, Nümbrecht, Germany). Directly before use, a 10 ml of 10% glucose solution (G-10, B. Braun, Melsungen, Germany) was added to 1 L of the CD and CDA solutions.

2.3. Experimental procedure

Mature domestic pigs (age = 13–15 weeks) were sedated using ketamine (30 mg/kg; i.m.) combined with azaperone (0.05 mg/kg; i.m.), and these pigs were anesthetized afterward with midazolam (0.1 mg/kg; i.v.) and ketamine (0.3 mg/kg; i.v.). This DCD model represents euthanasia without previous

TABLE 1 Composition of perfusion solutions.

	CD (Custodiol-N plus dextran)	CDA (Custodiol-N + dextran and albumin)	Steen™
Sodium	16	16	86
Potassium	10	10	4.6
Magnesium	8	8	0.8
Calcium	0.02	0.02	1.5
Chloride	30.04	30.04	
Histidine	124	124	
N-Acetylhistidine	57	57	
Sucrose	33	33	
α-Ketoglutarate	2	2	
Aspartate	5	5	
Glycine	10	10	
Alanine	5	5	
Tryptophan	2	2	
Arginine	3	3	
Deferoxamine (µmol/L)	25	25	
LK 614 (µmol/L)	7.5	7.5	
Dextran (g/L)	50	50	5
Albumin (g/L)		7	70
Glucose			11
Phosphate			1.2
pH	7.0[a]	7.0[a]	7.4[b]
Osmolarity (mOsm/L)	306	306	

All concentrations are given in mmol/L unless stated otherwise.
[a]At 20°C.
[b]Adjusted to pH 7.4 with sodium hydroxide.

treatment for lung protection because the pigs were neither heparinized nor ventilated during anesthesia. Sternotomy was performed after cardiac arrest (Maastricht category III) (14), and visible safe signs of death were observed as previously described (5, 15). Warm ischemia time was on average 60 min, and the ensuing lungs were then flushed antegradely and retrogradely with 4 L of 4°C cold low potassium dextran (LPD) solution (Perfadex™; XVIVO Perfusion, Gothenburg, Sweden) added with trometamol (1 mmol/L) and heparin (100 IU/L).

2.4. Experimental groups

Through a random selection process, four experimental groups were formed. The lungs were either prepared for the following processes: (i) I-EVLP ($n = 10$) after 1h of technical CSP followed by perfusion, or placement in standard preservation bags for 9h in 1L of 4°C LPD solution and subsequent perfusion (delayed perfusion). For the perfusion of the D-EVLP lungs, different acellular solutions were used: (ii) Steen solution (D-EVLP; $n = 8$) or modified Custodiol-N solution containing either (iii) dextran alone (D-EVLP/CD; $n = 8$) or (iv) dextran and albumin (D-EVLP/CDA; $n = 8$). The Toronto protocol (6) and the perfusion system XPS™ (XVIVO Perfusion, Gothenburg, Sweden) were used to perform 4 h of EVLP on all the four groups (**Supplementary Figure S1A**).

2.5. Cytokine and chemokine quantification

The perfusion solution samples were obtained at the beginning of perfusion (0 h) and after 1 and 4 h of perfusion. The samples were stored at −80°C. To quantify the concentration levels of 13 soluble molecules in the perfusates at the different time points, Luminex-based multiplex assay (Millipore porcine cytokine/chemokine 13-Plex, Merck, Darmstadt, Germany) was used according to the manufacturer's instructions. Standard curves and concentrations were calculated using the Bio-Plex Manager 6.1 software.

2.6. Oxygenation capacity measurement

Oxygenation capacity is defined as the difference between the pulmonary arterial pressure and the venous oxygen pressure. In this study, it was measured hourly during EVLP by blood gas analysis of the perfusates (ABL 700, Radiometer, Copenhagen, Denmark) at an FiO_2 of 1.0.

2.7. Statistical analyses

For descriptive statistical analyses, the GraphPad Prism software (version 9, La Jolla, CA, USA) was used. According to the D'Agostino–Pearson omnibus normality testing, the cytokine/chemokine concentrations were not normally distributed. Therefore, nonparametric two-tailed unpaired *t*-test (Kruskal–Wallis test) was used to compare the two groups. When comparing different time points between individuals of the different experimental groups, a paired *t*-test (Wilcoxon test) was used. For correlation analyses, Spearman test and linear regression were applied. Principal component analysis (PCA) plots and heatmaps were generated using the Qlucore Omics Explorer software (version 3.5, Lund, Sweden). Two-group or multigroup comparisons were used to identify cytokines/chemokines that differed most significantly between the experimental groups. Therefore, the data were $\log_2$ transformed and scaled to mean zero, variable one, and a threshold of 0.001. The statistical test used in each analysis and the *q*-value used as a cut-off are indicated in the figure legends. Significance was considered for *P*-values <0.05.

3. Results

To better understand the inflammatory reperfusion response induced during ischemia prior to implantation of the donor lung, we analyzed the cytokine/chemokine pattern in perfusates of the DCD lungs comparing different preservation protocols in a porcine model. The lungs were prepared for (i) immediate EVLP (I-EVLP) with Steen solution, (ii) cold storage for 9 h and subsequent EVLP with Steen solution (D-EVLP), (iii) subsequent EVLP with Custodiol-N solution added with dextran (D-EVLP/CD) alone, or (iv) subsequent EVLP with Custodiol-N solution added with dextran and albumin (D-EVLP/CDA; **Table 1**, **Supplementary Figure S1A**).

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3.1. D-EVLP and I-EVLP samples separate by perfusion time point rather than by CSP duration

To analyze for a potential effect of longer CSP times on the cytokine/chemokine pattern in the perfusates, we compared the I-EVLP samples with the D-EVLP samples. Interestingly, we found protein concentrations of the immediate and delayed EVLP samples to divide by perfusion time point rather than by CSP duration in PCA, displaying comparable cytokine/chemokine concentrations in perfusates of the groups using Steen perfusion solution (**Figure 1A**, **Supplementary Figure S1**). The samples obtained after 4 h of EVLP clearly separate from the other time points and generally show higher protein concentrations (**Figure 1A**). Unsupervised hierarchical cluster analyses (UHC) identified nine cytokines/chemokines with significantly different levels in perfusates between the start (0 h) and the end (4 h) of perfusion, namely, the pro-inflammatory cytokines/chemokines, such as TNF-α, IL-6, CXCL8/IL-8, IFN-γ, IL-12, IL-1α, and IL-1β, and IL-10 and IL-1RA, which are known to suppress the inflammatory response ($P = 0.01$; **Figure 1B**). Perfusates of the D-EVLP and I-EVLP group contained significantly higher concentrations after 4 h of perfusion of typical Th1 cytokines (TNF-α, IFN-γ, IL-12), Th2 cytokine IL-10, pro-inflammatory factors (IL-1α, IL-1β, IL-6, CXCL8), and the regulatory IL-1RA when compared with those found in the samples obtained at the beginning of perfusion (0 h) (INF-γ only for D-EVLP). Of note, TNF-α and CXCL8 levels were already significantly elevated after 1 h of immediate or delayed EVLP ($P < 0.05$). For all other analytes, the main increase in concentration was detected between 1 and 4 h of EVLP (**Figure 1C**), which indicates an intensified secretion of the proteins by the lung, reflecting the IRI.

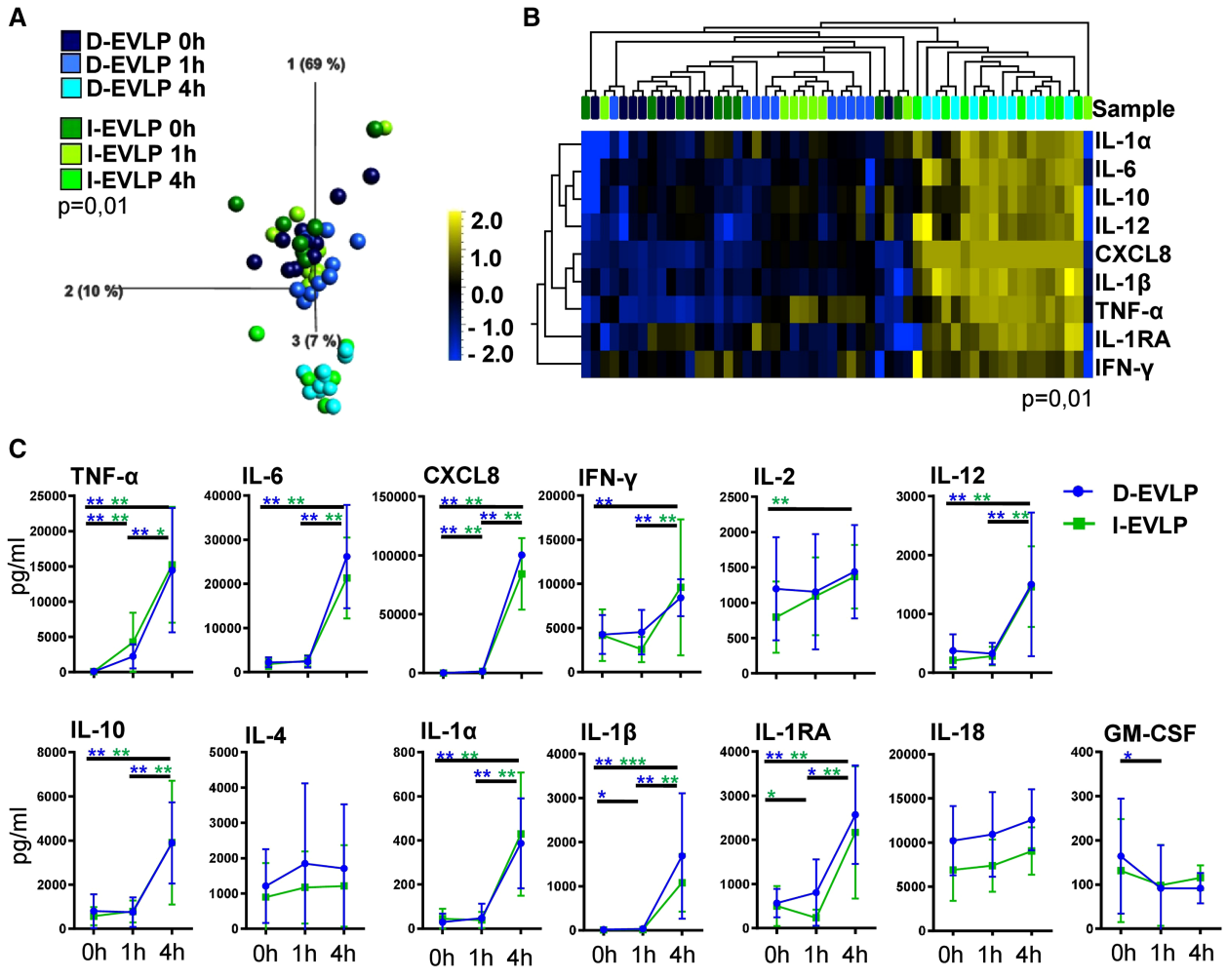

FIGURE 1
D-EVLP and I-EVLP samples separate by perfusion time point rather than by CSP duration. Perfusion samples were obtained directly at the beginning (0 h) and 1 and 4 h after perfusion and assessed for cytokine/chemokine secretion by Luminex-based multiplex assays. (**A**) Principal component analysis of the 13 cytokines according to delayed or immediate perfusion and time point ($P = 0.01$ and $q = 0.001$) and (**B**) unsupervised hierarchical clustering are shown. Two-group comparisons were used to identify variables differentially expressed between the two groups. Blue color indicates lower expression, and yellow color indicates higher expression. (**C**) Cytokine concentrations are displayed in time response for the two experimental groups. For statistical analysis, the different time points of each group were compared using a paired *t*-test (Wilcoxon). To compare the D-EVLP and I-EVLP groups at the different time points, a two-tailed, unpaired *t*-test (Kruskal–Wallis) was applied. Data are shown as mean ± SEM; asterisks indicate *P*-values with $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

3.2. D-EVLP/CD samples show a trend toward reduced protein secretion especially after 4 h of perfusion compared with the D-EVLP/CDA group

Elucidating whether albumin in addition to dextran affects the protein secretion of the allografts, we compared the cytokine/chemokine concentrations in D-EVLP/CD and D-EVLP/CDA perfusion samples. The samples were separated by analysis time point in PCA, with a significant increase in concentrations after 4 h of EVLP (Figure 2A). This finding indicates a comparable protein secretion and accumulation in the Custodiol-N groups. In line with this observation, the D-EVLP/CD and D-EVLP/CDA samples were clustered together, when comparing all four experimental groups (Supplementary Figure S1). Eight cytokines/chemokines were identified by UHC with significantly different levels in perfusates at the different sampling time points: the pro-inflammatory TNF-α, IL-6, CXCL8, IFN-γ, IL-12, and IL-1β and the anti-inflammatory IL-10 and IL-1RA ($P = 0.01$; Figure 2B). Interestingly, both UHC and PCA revealed a slight separation of the D-EVLP/CD and D-EVLP/CDA samples after 4 h of perfusion, showing the D-EVLP/CD samples trending toward lower concentrations when compared with the CDA samples.

Comparing the different analysis time points for the D-EVLP/CD and D-EVLP/CDA groups, we found significant differences in protein concentrations for both groups were detected between the 0 and 1 h time points for TNF-α and CXCL8. However, the main increases were observed when comparing the samples obtained at 4 h with the samples

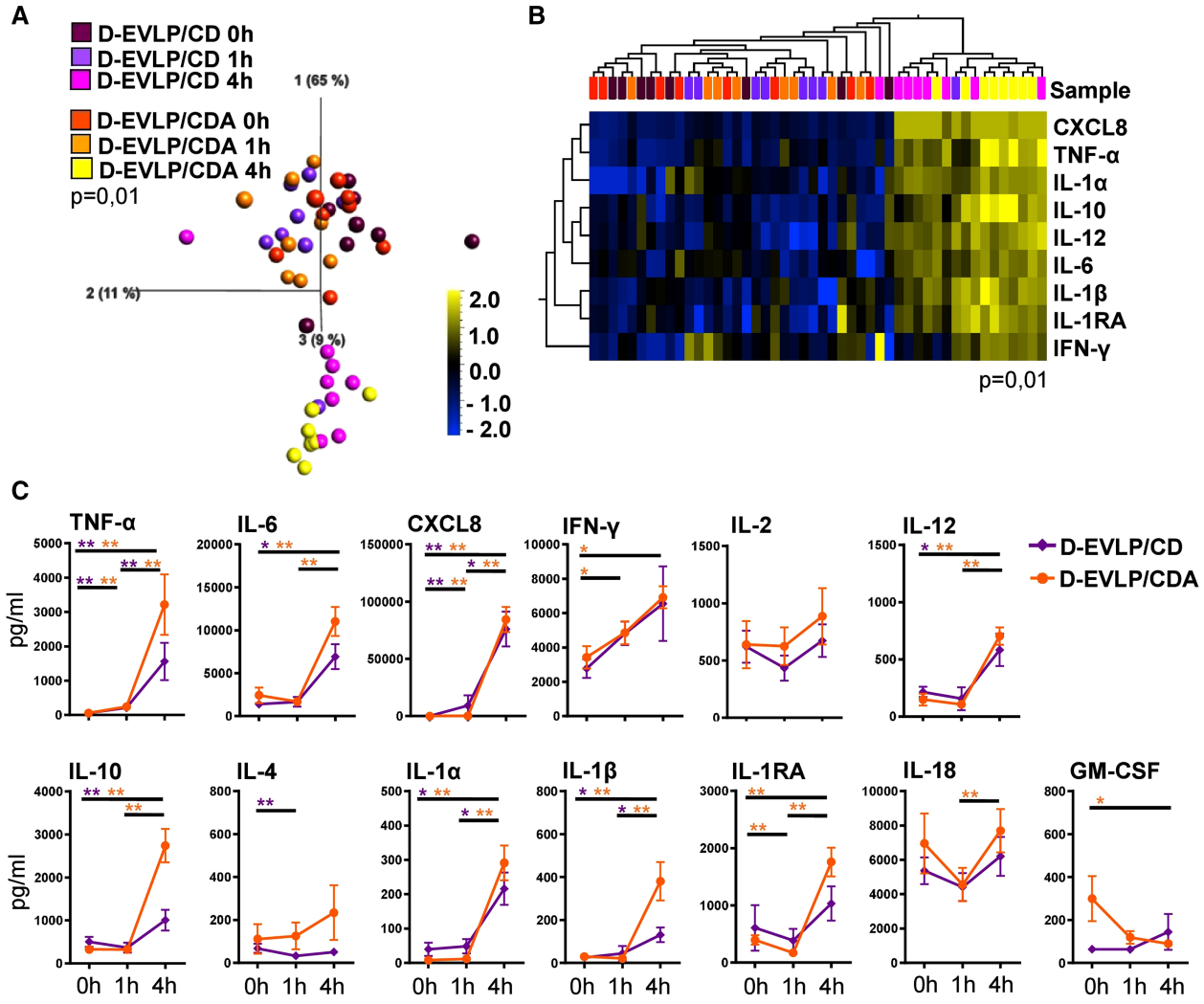

FIGURE 2
D-EVLP/CD and D-EVLP/CDA samples show separation by time point rather than by adding supplements to the perfusion solution. Samples were obtained and measured as described in Figure 1. (A) Principal component analysis of the 13 cytokines according to experimental groups and time point ($P = 0.01$ and $q = 0.004$) and (B) unsupervised hierarchical clustering are shown. Two-group comparisons were used to identify variables differentially expressed between the two groups. Blue color indicates lower expression, and yellow color indicates higher expression. (C) Cytokine concentrations are displayed in time response for the two experimental groups. For statistical analysis, the different time points of each group were compared using a paired *t*-test (Wilcoxon). To compare the D-EVLP/CD and D-EVLP/CDA groups at the different time points, a two-tailed, unpaired *t*-test (Kruskal–Wallis) was applied. Data are shown as mean ± SEM; asterisks indicate *P*-values with $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

obtained at earlier time points. Between 0 h and the end of perfusion (4 h) in both the D-EVLP/CD and D-EVLP/CDA groups, the concentrations of TNF-α, IL-6, CXCL8, IL-10, IL-12, IL-1α, and IL-1β were significantly elevated. An increase of protein concentration comparing 1 and 4 h time points in both groups was measured for TNF-α, CXCL8, IL-1α, and IL-1β. Remarkably, we detected a trend to lower concentrations and less increase in protein concentrations over time in the D-EVLP/CD group compared with the D-EVLP/CDA group. IL-1RA levels did not increase in the D-EVLP/CD group but were significantly elevated at 1 and 4 h in the D-EVLP/CDA group ($P < 0.01$; Figure 2C). These findings suggest less cytokine/chemokine secretion of D-EVLP/CD lungs when compared with the D-EVLP/CDA organs.

In conclusion, the D-EVLP/CD and D-EVLP/CDA samples showed no significant differences at the individual sampling time points, but a trend to lower protein concentrations in the D-EVLP/CD group was observed, indicating that there was no reducing effect of albumin on the protein secretion of the allograft.

3.3. Lowest cytokine/chemokine accumulation in the D-EVLP/CD group compared with all other experimental groups

In the next step, we dissected the differences in protein concentrations in the groups at 4 h of EVLP in more detail (Figure 3). The D-EVLP/CD samples reached significantly lower protein concentrations of TNF-α, IL-6, IL-12, IL-10, and IL-4 when compared with those in the delayed and immediate perfusion samples with Steen solution (I-/D-EVLP; $P < 0.05$; Figure 3). IFN-γ, IL-2, and IL-1RA were significantly less concentrated in the D-EVLP/CD samples compared with the D-EVLP (Steen) samples, but not in the I-EVLP samples. IL-1β concentrations displayed a slightly different pattern from the rest by showing significantly lower concentrations in the D-EVLP/CDA samples compared with the D-EVLP samples after 4 h of perfusion. Furthermore, IL-1β levels were significantly reduced in the D-EVLP/CD group compared with the I-EVLP group. Although significantly lower CXCL8 levels were quantified at 0 and 1 h of EVLP in the D-EVLP/CD and D-EVLP/CDA samples compared with Steen solution samples (Supplementary Figures S2A,B), no differences were detected after 4 h of EVLP, as all groups reached the highest measurable concentrations. To highlight the reduced cytokine/chemokine concentration in the D-EVLP/CD and D-EVLP/CDA groups, we performed a relative comparison of these experimental groups with the D-EVLP group. Therefore, the mean values were calculated for each group and time point. Subsequently, the mean values of the D-EVLP group were normalized to 100%, and relative percentages of the other groups were calculated. The most extensive reduction of concentrations after 4 h of perfusion was detected for IL-4 in the D-EVLP/CD group with only 3% of the compared D-EVLP concentrations. Also, IL-1RA, IL-1β, IL-2, IL-6, IL-10, IL-12, IL-18, and TNF-α have shown a reduction of >50% when comparing the D-EVLP/CD group with the D-EVLP group. The cytokine concentrations in perfusates using Custodiol-N solution supplied with dextran and albumin also displayed a relative reduction, but not as pronounced as in the D-EVLP/CD group (Supplementary Figure S3).

These findings suggest a reducing effect of Custodiol-N plus dextran, but not albumin, on the IRI and subsequent cytokine

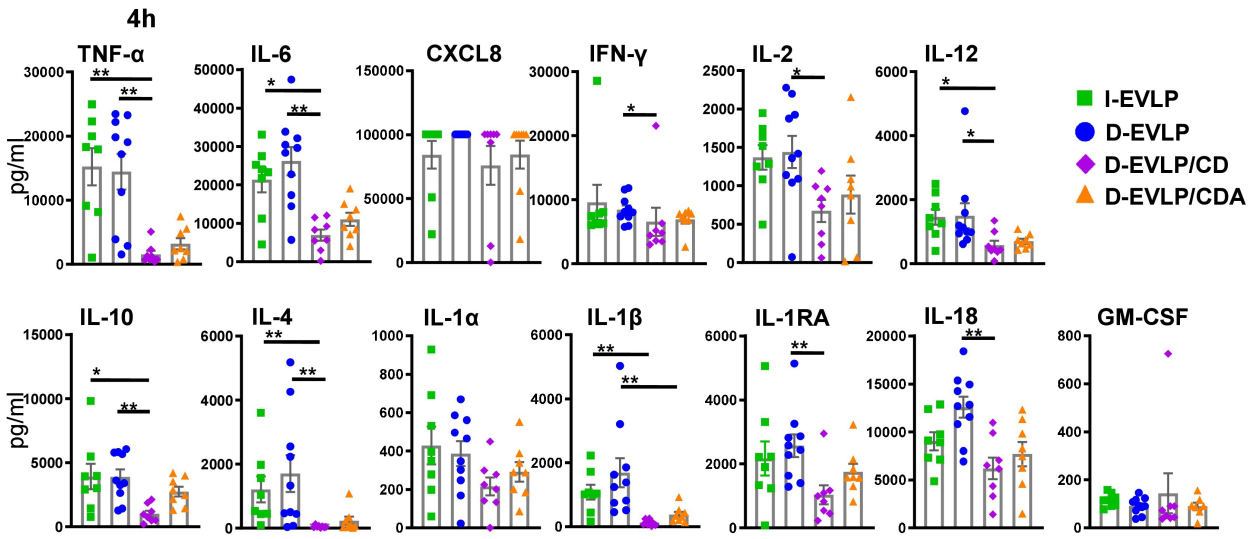

FIGURE 3
Lowest cytokine/chemokine accumulation in the D-EVLP/CDA group compared with all other experimental groups. The samples were obtained and measured as described in Figure 1. Cytokine/chemokine concentrations in the different perfusion solutions are displayed for each cytokine after 4 h of perfusion. For statistical analyses comparing the concentrations in the different experimental groups, a two-tailed, unpaired *t*-test (Kruskal–Wallis) was applied. Data are shown as mean ± SEM; asterisks indicate *P*-values with $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$; non-significant differences were not labeled specifically.

secretion of the allograft, resulting in less protein accumulation in perfusates after 4 h of EVLP.

3.4. Negative correlation of oxygenation capacities and cytokine/chemokine concentrations in perfusates

In order to reveal the potential effects of the different EVLP solutions on lung function, we correlated oxygenation capacities, measured in perfusates of all groups during EVLP, with the cytokine/chemokine concentrations. After 4 h of EVLP, higher oxygenation capacities were found to significantly correlate with lower concentrations of TNF-α, IL-6, IL-2, IL-12, IL-4, IL-1β, and IL-18 in all four experimental groups ($P < 0.01$; **Figure 4**). This negative correlation was evident for all measured cytokines/chemokines, except for granulocyte-macrophage colony-stimulating factor (GM-CSF), when including not only the 4 h time point, but also all the time points in the analysis ($P < 0.05$; **Supplementary Figure S4**). Taken together, these findings confirmed for EVLP to reduce IRI in the donor organs as evidenced by reduced cytokine/chemokine secretion.

4. Discussion

Ex vivo perfusion is a tool to preserve and improve especially marginal donor organs prior to transplantation. Despite major efforts, the optimal perfusion protocol and the precise composition of the perfusion solution in clinical LTX have not been identified. Our study aimed to investigate whether the CSP prior to EVLP (immediate *vs.* delayed) and the composition of the perfusion solution (Steen solution vs. Custodiol-N solution supplied with dextran or dextran/albumin) have impacts on the cytokine/chemokine secretion of the graft during EVLP and on the inflammation of the donor organ during the preservation process. We made use of an established surrogate porcine LTX model. The protein concentrations in perfusates were quantified at different time points during EVLP using Luminex-based multiplex assays and were then correlated to oxygenation capacities (12, 13).

We did not observe significant differences in the protein concentrations in perfusates (0 h) when comparing immediate and delayed EVLP after 1 and 9 h of CSP, respectively, indicating no further ischemic injury induced due to longer period of CSP. Interestingly, also after 1 and 4 h of perfusion, the protein concentrations in perfusates of the D-EVLP and I-EVLP samples remained comparable.

Consequently, longer period of CSP prior to EVLP seemed not to impact the cytokine/chemokine secretion of the graft during reperfusion. This is in line with previous findings, demonstrating that the pulmonary function of the harvested pig lungs was not impaired after a prolonged CSP when compared with immediate perfusion (12, 16). Of the analyzed proteins, only IL-18 showed a trend toward higher concentrations in perfusates of the D-EVLP samples, although not reaching significance. Similar observations were made in a porcine liver transplant model with elevated IL-18 concentrations in perfusate after a prolonged CSP (17), and IL-18 enhances the secretion of pro-inflammatory IFN-γ (18, 19). Remarkably, we did not detect increased IFN-γ concentrations in the delayed perfusion samples, suggesting that longer period of

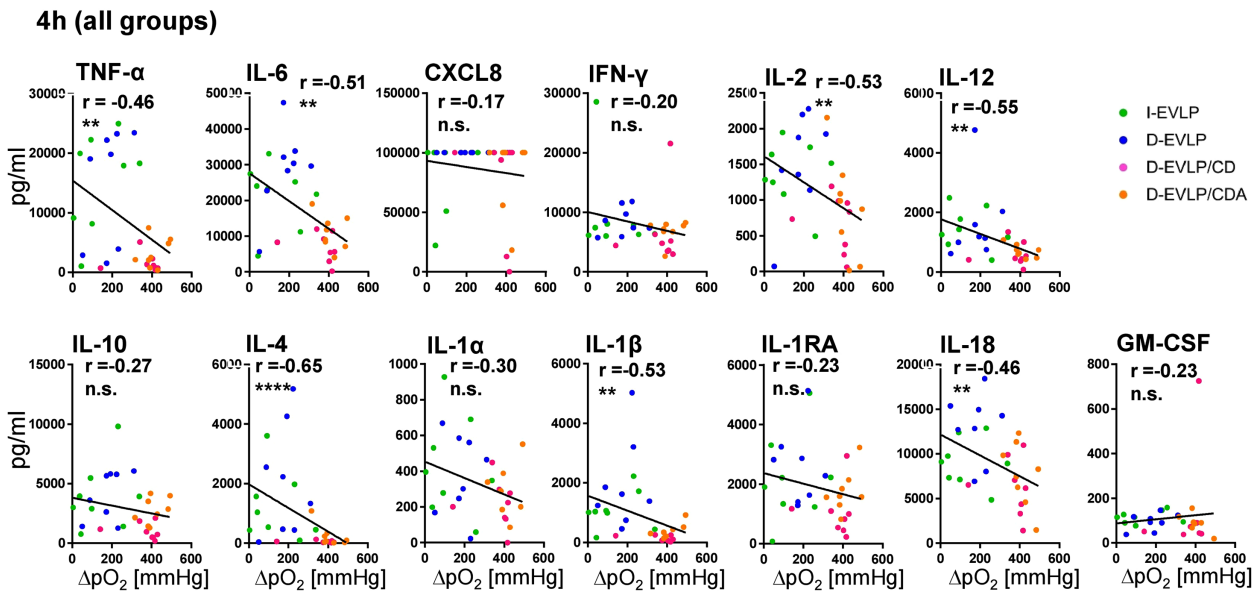

FIGURE 4
Negative correlations of oxygenation capacity and cytokine/chemokine concentrations in perfusates. Perfusion samples for cytokine/chemokine quantification were obtained and measured as described in Figure 1. Oxygenation capacity (ΔpO_2) was measured as described in the methods section. The different perfusates were labeled in colors; blue: D-EVLP; green: I-EVLP; pink: D-EVLP/CD; orange: D-EVLP/CDA. Correlation analyses (Spearman) of the oxygenation capacity and cytokine/chemokine concentrations after 4 h of perfusion were calculated. To highlight the negative correlation between these two variables, a linear regression of perfusion samples was performed. Asterisks indicate *P*-values with $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

CSP in the D-EVLP lungs did not translate into an enhanced secretion of inflammatory mediators (i.e., IFN-γ). Consequently, delayed *ex vivo* perfusion (9 h CSP) might represent a feasible strategy of lung preservation.

In addition, there could be beneficial effects of hypothermia on the donor organs, not only via suppression of pro-inflammatory mediators but also via enhanced secretion of tissue-protective cytokines such as IL-22 (20, 21). Moreover, lower expression of GSK-3β and reduced activation of NF-κB, two key players of the inflammatory cascade, were demonstrated in a rat model of hemorrhagic shock (22–24). Recent research focused on controlled hypothermia with stable temperatures of approximately 4°C–10°C to prevent freezing of the tissue. In this regard, it was shown that for heart and lung TX, the organs preserved with controlled hypothermia not only showed a normal perioperative function but also showed a reduced severity of primary graft dysfunction (21, 25, 26). However, our results have shown neither positive nor negative effects of longer period of CSP on the cytokine concentrations in perfusates.

Aside from the cold injury, the lung allograft is damaged upon reperfusion during EVLP and after implantation in the recipient not only through the inflammatory cascade involving immune cells but also through the response of both endothelial and epithelial cells. It was shown in several studies and in different organs that epithelial cells, as well as endothelial cells, are capable of secreting cytokines and chemokines after stimulation with danger signals or pro-inflammatory mediators (27, 28).

As a result of the ongoing IRI in the graft, cytokines/chemokines likely accumulate during 4 h of perfusion. Due to augmenting concentrations over time, the first hours of reperfusion rather than the duration of CSP seem crucial for the inflammation of the donor organ. Consequently, the aim is to optimize the reperfusion process, resulting in a reduced IRI. However, the analysis of the reperfusion upon implantation of the perfused donor organ was beyond the scope of our study. Although in a porcine model of LTX, lower cytokine/chemokine concentrations in perfusates (through EVLP combined with cytokine absorption) lead to an improved organ function even after TX (29).

Aside from the CSP duration, the chemical composition of the perfusion solutions could also affect the reperfusion process, hence potentially causing inflammation of the graft. The standard perfusion solution for EVLP is acellular Steen solution containing human serum albumin. However, in recent years, several alternative perfusion solutions have been developed, such as Custodiol-N, a modified cardioplegic solution designed for cold storage of solid organs (30–33). In contrast to the Steen solution, Custodiol-N contains the iron chelators deferoxamine and LK614, binding redox-active ions that might otherwise lead to the production of highly reactive oxygen species and subsequently lead to graft injury. A central role of iron ions in cold-induced cell injury (consisting of hypothermic injury and subsequent rewarming injury) and the protective effects of iron chelators were demonstrated in diverse cell types, including hepatocytes, endothelial cells, and cultured lung epithelial cells (34–36). Based on these observations, we hypothesize that Custodiol-N reduces the cytokine/chemokine secretion of the allograft during EVLP when compared with Steen solution, thereby reducing the inflammatory response of the transplanted graft. Therefore, another experimental group perfused with Custodiol-N, supplemented with Dextran40 (D-EVLP/CD) (33, 37), was included in our study. Along with maintaining colloid osmotic pressure, Dextran40 is known for its anti-thrombotic features and for protecting the endothelium from excessive leukocyte interactions by coating the endothelial surface (38). Finally, the perfusion solution of the fourth experimental group in our study was additionally supplemented with albumin (D-EVLP/CDA), which has been shown to stabilize and protect the endothelial glycocalyx by contributing to the endothelial surface layer (39).

Comparing cytokine/chemokine concentrations in perfusates, we observed significant differences depending on the perfusion solutions used. The groups employing Steen solution displayed higher concentrations of nearly all measured proteins when compared with groups perfused with Custodiol-N + Dextran40, with the difference being most pronounced after 4 h of perfusion. Endothelial protection by deferoxamine and LK614 possibly reduces the inflammatory reperfusion response in the lungs, although protection by other components of the solution and the protective effects on lung epithelial cells might also contribute to reducing inflammatory response. Moreover, lower concentrations of cytokine/chemokine could be the result of dextran inhibiting leukocyte interactions by coating and thereby protecting the endothelium. Yet, in the present study, it was not possible to assess the secretion of endothelial adhesion molecules (i.e., porcine intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM)) in the perfusates of the different experimental groups.

The beneficial effects of albumin on edema formation during heart perfusion were shown in a porcine study (40). Thus, we conceived that albumin could have a positive effect on the inflammation of the lungs by protecting the glycocalyx during the rewarming period. Contrary to our expectations, the addition of albumin to the perfusion solution had no reducing effect on cytokine/chemokine secretion during 4 h of EVLP since we did not detect significant differences in cytokine/chemokine concentrations at any sampling time point when comparing the D-EVLP/CD and D-EVLP/CDA groups. However, the D-EVLP/CD group showed a trend toward lower concentrations of the measured proteins. We lately reported slightly improved oxygenation capacities of pig lungs after 4 h of EVLP when perfused with Custodiol-N solution supplemented with dextran and albumin when compared with the addition of dextran only (13). The beneficial effects of albumin during prolonged period of EVLP might be due to improved oncotic support as evidenced by somewhat lower wet/dry weight ratios (13). Superior lung function in our cohort could result from the reduced inflammation due to elevated anti-inflammatory mediators (i.e., IL-10, IL-1RA) in the D-EVLP/CDA group.

Recently, it was shown that higher cytokine/chemokine levels in serum (i.e., IL-6, CXCL8) after transplantation are associated with worse clinical outcomes (41, 42). The correlation of the cytokine/chemokine concentrations with the oxygenation capacities of the lungs after 4 h of EVLP in our study revealed higher protein concentrations in perfusates to correlate with worse lung function. In line with our findings, it was shown that lower concentrations of pro-inflammatory IL-1β in perfusates are associated with better lung function 24 h post-transplantation (43). Moreover, cytokine

absorption prior to LTX resulted in an improved lung function, decreased inflammation, and less primary graft dysfunction in a porcine model, underlining the importance of reducing cytokine and chemokine concentrations during preservation (29, 44, 45). Therefore, D-EVLP/CD would provide the most usable donor organs for transplantation by reaching the lowest concentrations of cytokine/chemokine in the perfusate.

In our experimental groups, the D-EVLP/CD samples contained the lowest protein concentrations of e.g. the pro-inflammatory cytokines/chemokines CXCL8 and IL-6, known to be involved in the pathogenesis of primary graft dysfunction, and the elevated levels of CXCL8 in perfusates were suggested to predict the risk of the primary graft dysfunction (42, 46). Interestingly, the concentrations of the anti-inflammatory cytokines IL-10 and IL-1RA were also reduced in the D-EVLP/CD perfusate samples, suggesting a decreased secretion of pro- and anti-inflammatory mediators in the D-EVLP/CD group. Hence, the suppression of immune cells with hypothermia will reduce not only the pro-inflammatory cytokine secretion from these cells but also the anti-inflammatory cytokine secretion (47).

5. Conclusion

A prolonged CSP (9 h) does not affect the cold injury of the lung, while the first hours of reperfusion seem to be crucial for the damage of the allograft. Moreover, Custodiol-N supplied with dextran showed a reducing effect on the cytokine/chemokine secretion, while albumin exhibited no additional effect during 4 h of EVLP. The cytokine/chemokine concentrations in perfusates of Custodiol-N solution supplied with dextran and albumin negatively correlated with the oxygenation capacities, suggesting their usage as an alternative perfusion solution to the standard Steen solution. Possibly, by using Custodiol-N solution with dextran and albumin, more organs would reach the clinically relevant threshold of oxygenation capacity of >350 mmHg, and thus the transplant volume could be increased. Along with the composition of perfusate solutions, cytokine/chemokine concentrations in perfusates can be lowered by applying cytokine absorption filters. The latter were demonstrated to improve lung function of transplanted organs due to reduced IRI (29, 44, 45).

6. Limitations

The limitation of our study is focusing on the *ex vivo* perfusion period. The lungs were not transplanted, and there were no available data with regard to the post-perfusion period. Moreover, we only captured the oxygenation capacities as data displaying the lung function of the grafts.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Landesamt für Natur, Umwelt und Verbraucherschutz NRW. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LR performed the experiments, analyzed the data, and wrote major parts of the manuscript. AK, CO, and YL performed the animal experiments. KB and JK performed Luminex-based multiplex experiments, and UR provided perfusion solutions. JFK helped in analyzing the data and wrote major parts of the manuscript. CF and MK supervised the work, designed the experiments, analyzed the data, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor CA declared a past co-authorship with the author CF.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2023.1245618/full#supplementary-material

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Proteomic changes to immune and inflammatory processes underlie lung preservation using *ex vivo* cytokine adsorption

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Introduction: In recent years, the field of graft preservation has made considerable strides in improving outcomes related to solid organ restoration and regeneration. Ex vivo lung perfusion (EVLP) in line with the related devices and treatments has yielded promising results within preclinical and clinical studies, with the potential to improve graft quality. Its main benefit is to render marginal and declined donor lungs suitable for transplantation, ultimately increasing the donor pool available for transplantation. In addition, using such therapies in machine perfusion could also increase preservation time, facilitating logistical planning. Cytokine adsorption has been demonstrated as a potentially safe and effective therapy when applied to the EVLP circuit and post-transplantation. However, the mechanism by which this therapy improves the donor lung on a molecular basis is not yet fully understood.

Methods: We hypothesized that there were characteristic inflammatory and immunomodulatory differences between the lungs treated with and without cytokine adsorption, reflecting proteomic changes in the gene ontology pathways and across inflammation-related proteins. In this study, we investigate the molecular mechanisms and signaling pathways of how cytokine adsorption impacts lung function when used during EVLP and post-transplantation as hemoperfusion in a porcine model. Lung tissues during EVLP and post-lung transplantation were analyzed for their proteomic profiles using mass spectrometry.

Results: We found through gene set enrichment analysis that the inflammatory and immune processes and coagulation pathways were significantly affected by the cytokine treatment after EVLP and transplantation.

Conclusion: In conclusion, we showed that the molecular mechanisms are using a proteomic approach behind the previously reported effects of cytokine adsorption when compared to the non-treated transplant recipients undergoing EVLP.

KEYWORDS

lung transplantation, mass spectrometry, *ex vivo* lung perfusion, cytokine adsorption, proteomics

Introduction

The improvement of graft preservation remains a key goal in lung transplantation, with the ultimate objective of increasing the number and quality of transplants. Graft preservation is a key topic given the necessity of maintaining the quality of the harvested organ after it has been removed from the donor and remains in a vulnerable state, subject to ischemic damage. *Ex vivo* lung perfusion (EVLP) is a platform for evaluating and potentially treating donor lungs using machine perfusion with ventilation and perfusion support. This system was initially used for evaluating lung function in lungs from uncontrolled donation after circulatory death and later for evaluating marginal donor lungs, with the Lund group performing the first transplant using marginal lungs after EVLP in 2005 (1–5). Subsequent development has allowed for a stable EVLP protocol that can run for hours and evaluate suboptimal lungs (6, 7). The system also allows for implementing targeted therapies that could be used to recondition or ameliorate marginal and damaged lungs (8). The benefits of delivering a specific intervention during EVLP include using an isolated system in which the treatment can be administered directly to the target organ, bypassing any effects treatment may have in the systemic circulation.

The restoration or regeneration of damaged organs is a particularly important goal given the number of lungs that are declined for transplantation due to poor quality. Donor organ availability limits the number of possible transplantations, resulting in waiting list mortality. An estimated 60% of donor lungs are rejected after evaluation for acceptance, with the fear that damaged lungs will result in higher complication rates (9, 10). Refusal of lung grafts can caused by acute lung injury (ALI) stemming from several etiologies, such as aspiration, infection, trauma, and neurogenic edema, with the most severe form of ALI manifesting as acute respiratory distress syndrome (ARDS) (11).

Thus, EVLP allows for treating lungs that are damaged by ALI and ARDS to recuperate them for transplantation. Several potential therapies have been tested for graft preservation using EVLP, such as the use of mesenchymal stem cells, dialysis, and cytokine adsorption, to name a few. In particular, cytokine adsorption has been investigated given the established significance of cytokines in mediating inflammatory processes. Adsorbers rely on polymer beads that target the middle and low-molecular-weight molecules, which have been shown to reduce cytokine levels in severe sepsis (12–16). They have also been used in other transplantation types, such as orthotopic heart and kidney transplants (17, 18). We previously reported on the use of cytokine adsorption in a porcine model of transplantation (19) and human transplants (20). In recipients with lungs treated during EVLP and post-transplantation, inflammation and pulmonary function improved, and the incidence of primary graft dysfunction was reduced. The mechanisms and pathways involved in graft improvement with cytokine adsorption both from this and other EVLP studies have not been fully elucidated. Data on the proteomic profiles characterizing lung transplantation using tissue are extremely limited, particularly from models after the transplant in the recipients and none as far as we identified specific to cytokine adsorption (21, 22). We hypothesized that a proteomic approach would demonstrate what pathways were altered by the treatment. Using lung biopsies obtained during EVLP and post-transplantation in our model, we compared the treated grafts to the non-treated grafts and compared both to the normal lungs, finding significant alterations to pathways related to immune and inflammatory processes.

Methods

Porcine model

Ethical considerations for porcine experiments

The study was approved by the local Ethics Committee for Animal Research (DNR 5.2.18-4903/16 and DNR 5.2.18-8927/16) of the Lund University. All animals received care according to the USA Principles of Laboratory Animal Care of the National Society for Medical Research, Guide for the Care and Use of Laboratory Animals, National Academies Press (1996).

Animal preparation

A total of 24 male and female adult farm-raised wild-type American Yorkshire pigs (*Sus scrofa domesticus*) were used in this study, with 12 designated as donors and 12 designated as recipients. These animals were previously described in a prior publication, which includes alternate variables and outcomes that were not re-reported in this publication (19). Seraclone™ Anti-A blood grouping reagent (Bio-Rad, Medical Diagnostics GmbH, Dreieich, Germany) was used to determine the blood types of animals prior to the experiment, and these animals were then matched as donor–recipient pairs according to blood type and weight. Premedication, preparation, and ventilatory settings were previously described (19), such as xylazine (Rompun vet. 20 mg/kg, Bayer AG, Leverkusen, Germany), ketamine (Ketaminol vet. 20 mg/kg, Farmaceutici Gellini S.p.A., Aprilia, Italy), peripheral line insertion, urinary catheter, and endotracheal intubation. Mechanical ventilation (Servo 900C, Siemens, Solna, Sweden) was set to volume-controlled ventilation with a 1:2 inspiration-to-expiration ratio and a tidal volume of 6–8 ml/kg. All animals were monitored with a pulmonary artery catheter (Swan-Ganz CCOmbo V and Introflex, Edwards Lifesciences Services GmbH, Unterschleissheim, Germany).

ARDS induction in donor animals

ARDS was induced using lipopolysaccharide (LPS) from the Gram-negative bacterium *Escherichia coli* (O111:B4, Sigma Aldrich, Merck KGaA, Darmstadt, Germany), as previously described (19). The endotoxin was administered as an infusion diluted in saline over 1 h (2 μg/kg/min) and then at a lower rate for another 1 h. All animals developed hemodynamic instability,

which was treated with a continuous infusion of norepinephrine (40 μg/ml, 0.05–2 μg/kg/min) (Pfizer AB, Sollentuna, Sweden) and dobutamine (2 mg/ml, 2.5–5 μg/kg/min) (Hameln Pharma Plus GmbH, Hameln, Germany). Fluid loss was compensated with Ringer's acetate (Baxter Medical AB, Kista, Sweden). A total volume of 6 ml of blood sample was collected before induction of lung injury and thereafter every hour from the injured donor who was used for the analyses as previously reported (19). In addition, 1 ml of blood sample was collected for blood gas measurements every 30 min.

The Berlin criteria were used to define the ARDS stage based on the PaO_2/FiO_2 ratio taken using arterial blood gases. Blood gases were analyzed every 30 min (ABL 90 FLEX blood gas analyzer, Radiometer Medical ApS, Brønshøj, Denmark) and were normalized to a blood temperature of 37°C. Pulmonary harvest only proceeded after two arterial blood gases taken 15 min apart demonstrated a PaO_2/FiO_2 ratio of less than 300 mmHg and an absence of heart failure.

Pulmonary harvest, EVLP, and left lung transplantation in the recipients

Pulmonary harvest, EVLP, and transplantation have been previously described in detail (19). In brief, pulmonary harvest was performed according to clinical practice and proceeded in an *en bloc* fashion through a median sternotomy and cannulation of the pulmonary artery and clamping of the superior vena cava, inferior vena cava, and ascending aorta. The lungs were anterogradely flushed with cold Perfadex® PLUS solution (XVIVO perfusion, Gothenburg, Sweden).

The *en bloc* lungs were placed on a Vivoline LS1 (XVIVO perfusion, Gothenburg, Sweden) for EVLP with a target perfusion of 40% of cardiac output and a tidal volume of 7 ml/kg of the donor body weight (23, 24). The system was primed with Steen™ Solution (XVIVO perfusion) and set to a circuit hematocrit of 15%–20% using donor red blood cells collected prior to ARDS induction. An additional Steen solution was added when the perfusate levels in the reservoir dropped below 300 ml. The lungs were cooled to 8–12°C for approximately 45 min before transplantation.

The left lung transplantation was performed according to clinical practice and as previously described, including the post-transplantation follow-up and immunosuppression (19). All animals were immunosuppressed using tacrolimus (0.15 mg/kg, PO) (Sandoz AS, Copenhagen, Denmark) and methylprednisolone (1 mg/kg, intravenously, twice daily) (Solumedrol, Pfizer, New York, USA). Bronchoscopy was used to confirm an open bronchial anastomosis.

Recipient follow-up and right pneumonectomy on the third day post-transplantation

The animals were kept under anesthesia for 3 days as previously described in detail (19). The recipient ventilatory strategy included the use of the lowest possible pressures while maintaining adequate oxygenation and ventilation with a positive end-expiratory pressure between 5 and 10 cmH_2O and a peak pressure below 30 cmH_2O. Arterial blood gases were monitored every hour post-transplantation. The pulmonary hilum was dissected through a mid-sternotomy, and a pneumonectomy of the right lung and accessory lobe allowed for an isolated assessment of the transplanted left lung. The recipient was followed for an additional 4 h during single-lung ventilation, and the tidal volume and respiratory rate were adjusted to maintain a peak pressure <30 cmH_2O.

Treatment with cytokine adsorption

As previously described (19), an adsorbent filter (CytoSorb®, CytoSorbents Europe GmbH, Berlin, Germany) was used to continuously filter the perfusate during EVLP through a veno-venous shunt from the reservoir at a rate of 300 ml/min. The filter was in place throughout EVLP and was followed up post-transplantation with an additional 12 h of extracorporeal hemoadsorption connected to the cytokine adsorber via a veno-venous shunt using a hemodialysis catheter (Power-Trialysis® Slim-Cath™, Becton, Dickinson and Company, NJ, USA) inserted in the jugular vein. The roller pump ran at a rate of 300 ml/min (19).

Mass spectrometry analysis

Sample preparation and protein digestion

Biopsies were taken from the right lung after intubation as baseline samples with random selection from both the treated and non-treated groups ($n = 6$ baseline samples). Biopsies were also obtained from the right lung after 4 h of EVLP and from the left lung post-transplantation on day 3 of observation from both the treated and non-treated groups ($n = 6$ per baseline, treated EVLP, non-treated EVLP, and non-treated post-transplant groups and $n = 5$ for treated post-transplant group after analysis). Proteins were extracted from homogenized tissues and were solubilized in a 2% sodium dodecyl sulfate (SDS, Sigma Aldrich, Darmstadt, Germany). A bicinchoninic acid (BCA) assay (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) was performed to determine protein concentration. Subsequently, 30 μg of protein was digested using an S-Trap digestion protocol. Samples were reduced with 20 mM dithiothreitol (DTT, Sigma Aldrich) for 45 min at 56°C and then incubated with 40 mM iodoacetamide (IAA, Sigma Aldrich) in the dark at room temperature for 30 min. Samples were acidified with 2.5% phosphoric acid and washed with buffer before binding to an S-Trap CO_2-micro-80 column (ProTifi, Fairport, New York, USA) and were double digested overnight at 37°C with lysine-C (Promega mass spec grade at a 1:50 ratio of enzyme to protein by ng, Promega, Madison, WI, USA) and trypsin (Promega sequence grade at a 1:50 ratio of enzyme to protein by ng).

Peptide mixing and pre-fractionation

Fractionation was carried out using a Waters XBridge BH130 C18 3.5 µm, 2.1 mm × 150 mm column, on an Ultimate 3000 rapid separation high-performance liquid chromatography (RS HPLC) (Thermo Scientific, Waltham, MA, USA) operating at 200 µl/min. The mobile phases were solvent A, 10 mM ammonium formate at pH 10, and solvent B, 90% acetonitrile and 10% water containing 10 mM ammonium formate at pH 10. Peptides were separated using the following gradients: 0 min 0% B; 3 min 0% B, 97 min 35% B; 98 min 80% B; and 108 min 80% B. The column was operated at RT, and the detection wavelength was 214 nm. We collected 96 fractions at 1 min intervals that were further concatenated to 48 fractions by combining two fractions that are 24 fractions apart, i.e., #1 and #25 and #2 and #26. The fractions were dried in the SpeedVac.

LC-MS/MS data acquisition

DDA data acquisition on timsTOF Pro 2

The fractions were resuspended in 0.1% formic acid, and peptide determination was performed in a NanoDrop system (DeNovix, Wilmington, DE, USA) before liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. A total of 400 ng of each fraction was loaded on Evosep tips (Evosep Biosystems, Odense, Denmark) for separation with nanoflow reversed-phase chromatography with an Evosep One LC system (Evosep Biosystems). Separation was performed with the 30 SPD method (gradient length of 44 min) using a 15 cm × 150 µm Evosep column (Evosep Biosystems) packed with 1.5 µm ReproSil-Pur C18-AQ particles. The Evosep One was coupled to a timsTOF Pro 2 ion mobility mass spectrometer (Bruker, Billerica, MA, USA) operated in data dependent acquisition - parallel accumulation - serial fragmentation (DDA PASEF) with 10 PASEF scans per acquisition cycle and accumulation and ramp times of 100 ms each. Singly charged precursors were excluded, the "target value" was set to 20,000, and dynamic exclusion was activated and set to 0.4 min. The quadrupole isolation width was set to 2 Th for $m/z < 700$ and 3 Th for $m/z > 800$. All subsequent DDA files were used to build a spectral library in Fragpipe v 180 (25–28), with the following parameters, i.e., missed cleavages = 2, min peptide length = 7, max peptide length = 50, and common internal retention time peptides (CiRTs), that were used for spectral library retention time calibration. UniProt UP000008227 FASTA (release 2023_01) was used as a database with reversed target sequences as decoys. The generated library consisted of 10,296 protein groups in total. A py_diAID method (29) was generated by subjecting the 48 DDA fraction runs and was used to run all individual samples in the study in diaPASEF on the timsTOF Pro 2.

DIA data acquisition on timsTOF Pro 2

The samples were loaded onto Evosep tips (Evosep Biosystems) and separated with the same gradient as for DDA data acquisition. MS data were acquired using the diaPASEF method. The accumulation and ramp times were set to 100 ms. DIA scans were acquired with 25 m/z isolation windows spanning 247–1,350 m/z and 0.60–1.60 1/K0 ion mobility ranges and an estimated cycle time of 2.76 s. The collision energy was ramped linearly as a function of the mobility from 59 eV at 1/K0 = 1.6 Vs cm^{-2} to 20 eV at 1/K0 = 0.6 Vs cm^{-2}.

Bioinformatic analysis of LC-MS/MS data

Raw LC-MS/MS data were analyzed using DIA-NN v 1.8.1. The quantification mode was set to Robust LC (high precision), with default RT-dependent normalization and the Fragpipe spectral library. Subsequently, the output files were loaded into RStudio v 2022.12.0 with R v 4.2.2. The MS-DAP package v 1.05 (30) was used for normalization and differential expression analysis. The identified proteins were first filtered at 75% of identifications per contrast. Normalization was performed using variance stabilizing normalization followed by mode between protein normalization. Differential expression analysis was performed using the DEqMS R package (31). Log(2)-fold change thresholds were inferred through bootstrapping in the MS-DAP package. Significantly changed proteins were defined as false discovery rate (FDR)-corrected p-values (q-values) of >0.05 and Log (2)-fold change of ±0.313, 0.344, and 0.354 for the non-treated vs. treatment, baseline vs. treatment, and baseline vs. non-treated groups, respectively. For the heatmap, the MaxLFQ values were normalized using z-scores and plotted using the pheatmap package v 1.0.12 with Euclidean clustering. Gene set enrichment analysis (GSEA) analysis was performed by examining all proteins found through bioinformatic analysis after the filtering and normalization steps using the clusterProfiler package v 4.4.4. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD044413.

Calculations and statistics

Continuous variables were reported as median and interquartile range (IQR). Statistically significant differences between groups were tested with the Student's t-test when comparing the two groups and within groups using ANOVA when data were normally distributed. Most analyses were conducted with the Mann–Whitney test and the Kruskal–Wallis tests when data were not normally distributed. A chi-squared test was performed to analyze the observed frequencies of the categorical variables. These statistical analyses were performed using GraphPad Prism 9.1. The statistics used within mass spectrometry analysis are reported in the "Bioinformatic analysis of LC-MS/MS data" section. Significance was defined as $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*), and $p > 0.05$ (not significant).

Results

Mass spectrometry analysis showed significant differences in protein expression after cytokine adsorption treatment in EVLP

Lung biopsies obtained prior to injury induction were collected as baseline samples and subsequently compared to the lung biopsies obtained after EVLP from both the treated and non-treated groups. After induction of lung injury, we found no significant differences in the PaO_2/FiO_2 ratios between the treated (208.2 ± 55.5 mmHg) and non-treated groups (225.3 ± 33.6 mmHg, $p = 0l733$). By the end of EVLP, the treated lungs had increased their ratio to 324 ± 70 mmHg, whereas the non-treated lungs did not pass the clinical acceptance with a PaO_2/FiO_2 ratio of 249 ± 143 in the 10 pigs assigned to the non-treated group.

Figure 1A shows the volcano plots depicting the upregulated and downregulated proteins across the three comparison types. In the comparison between the baseline and the non-treated groups, 5,399 proteins were identified, of which 620 were statistically differentially expressed. Of those proteins that were differentially expressed, 373 were downregulated and 247 were upregulated. In the comparison between the baseline and the treated groups, 164 proteins were downregulated, and 157 were upregulated. Furthermore, in the comparison between the non-treated and treated groups, 57 proteins were downregulated and 112 were upregulated. Within the three groups, an unsupervised hierarchical clustering that was performed on the differentially expressed proteins demonstrated clustering of the groups (Figure 1B). The baseline, treated, and non-treated groups were discretely sorted based on the clustering of the proteins, showing the significantly different protein expression profiles at this point in EVLP.

In examining the clustering of protein expression profiles further, several comparison groups were considered separately and displayed to the left of the heatmap. This includes the significantly differentially expressed proteins found in the comparisons between the baseline and non-treated samples (yellow column, Figure 1B) and those between the baseline and treated samples (green column, Figure 1B). Of those proteins, the overlap in identities is displayed in the Venn diagram, as shown in Figure 1C. In addition, the specific pathways identified from the GSEA were highlighted to demonstrate the distribution of identified proteins across their locations in the heatmap (Figure 1B). Specifically, the immune system process pathway [gene ontology (GO) term GO:0002376] is displayed in the red column and blood coagulation (GO:0007596) in the purple column (Figure 1B). The immune system process term consists of a protein set size of 276 proteins and had a normalized enrichment score (NES) of 1.54 (q-value 0.028) when comparing the baseline and non-treated samples. Within the baseline and treated samples, the NES of the immune system process was 1.72 with a q-value of 0.027. For the term blood coagulation, when comparing the baseline group and the non-treated group, the pathway had an NES of −2.11 and a q-value of 0.002, and when comparing the baseline group and the treated group, the pathway had an NES of −1.84 and a q-value of 0.09.

GSEA analysis across the comparisons between the baseline vs. non-treated and baseline vs. treated groups yielded enriched pathways, as shown in Figure 2. The hierarchical clustering was performed on the GO terms to demonstrate the overarching biological processes underscored by the GO terms. The clustering of enriched terms relied on pairwise similarities of the terms, and these terms were divided largely into five groups. These groups included mainly immune responses and responses to external stimuli, cell motility terms, coagulation terms, lipid transport, and leukocyte immunity, respectively.

Individual proteins were identified for comparison across all three groups (Figure 3 and Table 1). Proteins related to inflammation and cytokine processes were found to be significantly increased in the non-treated group compared to those in the baseline group. These proteins include toll-like receptor 2 [TLR2, log(2)-fold change 1.14, q-value 0.007], apoptosis-associated speck-like protein containing a CARD [PYCARD, log(2)-fold change 0.84, q-value 0.005], and chemerin-like receptor 1 [CMLK1, log(2)-fold change 1.64, q-value 0.0005]. The interleukin-1 receptor accessory protein (IL-1 RAP) was numerically higher in the treated and non-treated groups, but this was not a statistically significant elevation [log(2)-fold change 0.50, q-value 0.25].

Several comparisons were not statistically significantly different but showed a numerical change between the treated and non-treated groups, such as the interleukin-1 receptor antagonist protein [IL-1 RN, log(2)-fold change −0.27, q-value 0.41], pro-interleukin-16 [IL-16, log(2)-fold change −0.22, q-value 0.24], and TLR 2 [log(2)-fold change −0.06, q-value 0.86]. Other observed numerical decreases in expression included PYCARD [log(2)-fold change −0.33, q-value 0.17], interleukin-6 cytokine family signal transducer [IL-6ST, log(2)-fold change −0.08, q-value 0.83], chemerin-like receptor 1 [CMLK1, log(2)-fold change −0.22, q-value 0.42], and REL proto-onco [REL, log (2)-fold change −0.19, q-value 0.57].

In addition, numeric, though not statistically significant, decreases in proteins related to neutrophils (Figure 4) between the treated and non-treated groups were found. These proteins include azurocidin [AZU1, log(2)-fold change −0.08, q-value 0.92], proteinase 3 [PRTN3, log(2)-fold change −0.55, q-value 0.18], myeloperoxidase [MPO, log(2)-fold change −0.003, q-value 0.99], protein S100A8 [log(2)-fold change −0.21, q-value 0.69], dipeptidyl peptidase 1 [DPP1, log(2)-fold change −0.28, q-value 0.07], and neutrophil-related elastase [ELANE, log(2)-fold change −0.15, q-value 0.76]. Moreover, there were numeric decreases in proteins related to macrophage-related proteins, such as CD163 [log(2)-fold change −0.48, q-value 0.13] and nitric oxide synthase [NOS, log(2)-fold change −0.20, q-value 0.66]. This coincided with an increase in the nitric oxide synthase trafficking protein when comparing the treated and non-treated values [nitric oxide synthase trafficking inducer (NOSTRIN), log(2)-fold change 0.45, q-value 0.07].

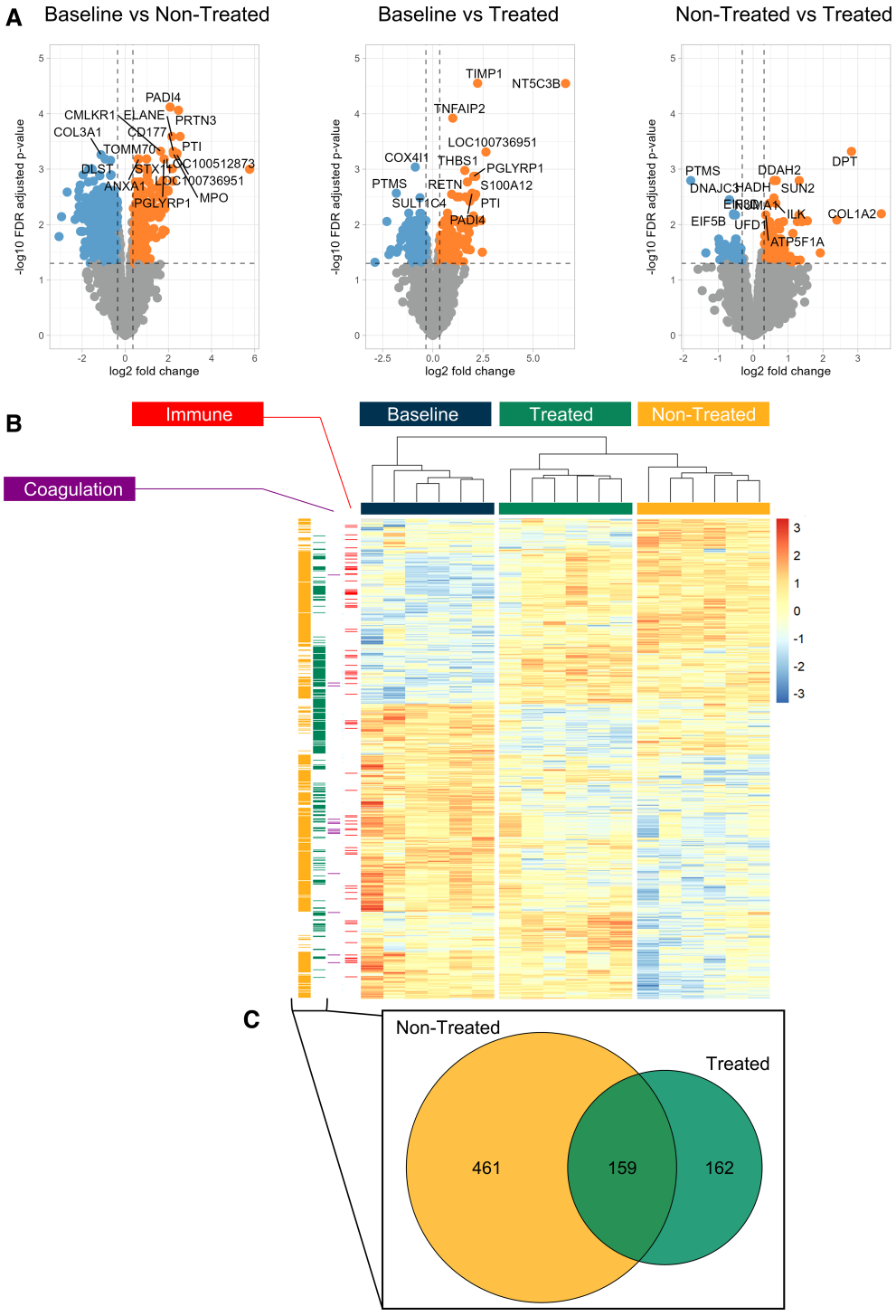

FIGURE 1
Treated and non-treated lungs could be clearly distinguished by their proteomic profiles based on differential expression of proteins found at the end of EVLP. Comparisons between the groups and those with normal lung tissue (baseline) showed significant differences in the overall distribution of protein expression. (**A**) Volcano plots of the proteins identified across the three comparison groups (non-treated vs. treated, baseline vs. treated, and baseline vs. non-treated). For those that were statistically significantly differentially expressed, blue marks the underexpressed proteins, and orange marks the overexpressed proteins within the comparisons. (**B**) Unsupervised hierarchical clustering was performed on the proteins to produce the heatmap across the three groups. GSEA showed pathways from the biological processes. The proteins from GO terms are highlighted in columns to the left of the heatmap: the immune system process pathway (red, GO Term GO:0002376) and coagulation (purple, GO:0007596). The proteins that were differentially expressed in the non-treated group compared to the baseline group are shown separately in the yellow column to the left of the heatmap. The differentially expressed proteins in the treated group are shown in the green column to the left. (**C**) Of those proteins that were differentially expressed between baseline vs. treated groups and baseline vs. non-treated groups, the Venn diagram demonstrates the distribution of unique or shared identities. All graphs represent data from the treated group ($n = 6$), non-treated group ($n = 6$), and baseline group ($n = 6$). Statistically significant differences are reported as FDR-corrected p-values (q-values) using log(2)-fold change differences between groups.

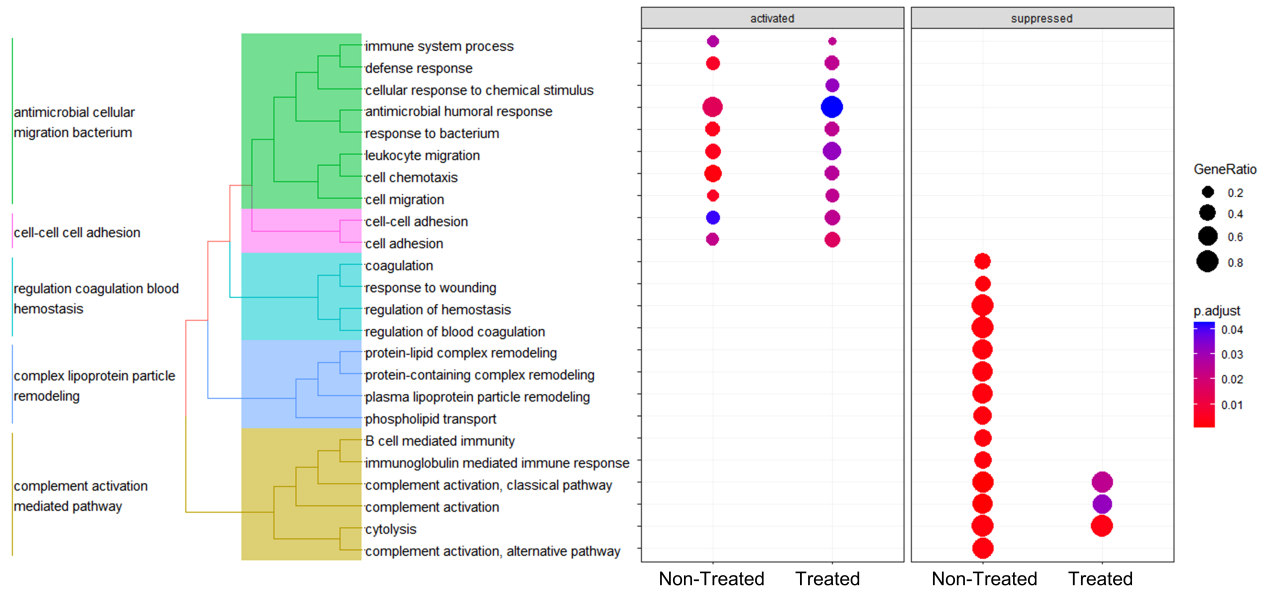

FIGURE 2
Key responses to injury were enriched in the treated and non-treated groups using GSEA during EVLP. Enriched pathways from the GSEA are shown for both the non-treated and treated groups in the dot plot. Unsupervised hierarchical clustering of the GO terms was performed to show the overarching biological processes involved. All graphs represent the data from the treated group ($n = 6$), non-treated group ($n = 6$), and baseline group ($n = 6$). Statistically significant differences are reported as FDR-corrected p-values (q-values) using log(2)-fold change differences between groups.

Pathways related to the immune system and inflammatory responses were significantly altered in the treated group after lung transplantation

Samples collected from the third day after the transplantation were compared between the non-treated and treated groups and additionally analyzed compared to the same baseline samples as previously described. By the end of the experiment, the PaO_2/FiO_2 ratio of the treated group (442.0 ± 90.2 mmHg) was significantly higher than that of the non-treated group (174.9 ± 31.0 mmHg, $p = 0.0022$).

Based on the expression profiles of the identified proteins, the hierarchical clustering again showed the separation of the groups into baseline, treated, and non-treated groups (Figure 5A). The same immune system process pathway and coagulation pathway were highlighted in separate columns to the left of the heatmap to demonstrate the location of the proteins identified within these sets. When comparing the treated and non-treated groups, the immune system process had an NES of −1.78 with a q-value of 5.22×10^{-8}, whereas the stress response had an NES of 1.65 and a q-value of 5.21×10^{-8}. Furthermore, the proteins differentially expressed in the baseline to non-treated comparison (yellow column, Figure 5A) and the baseline to treated comparison (green column, Figure 5A) were placed adjacent to the heatmap to show protein locations. From the expressed proteins, a greater extent of identity overlaps with 582 proteins in common can be found between the baseline to non-treated and baseline to treated comparisons, as shown in the Venn diagram in Figure 5B.

GSEA analysis of the proteins found within the comparisons showed a number of similar pathways as found within the EVLP. Several pathways were identified as either activated or suppressed within the non-treated vs. treated comparison (Figure 6). The only statistically significantly activated process was the term "toxin metabolic process." All others were suppressed in the treated group, which included pathways involving immune responses, stress responses, wound healing, and defense responses. In addition, blood coagulation and hemostasis were identified as suppressed pathways.

The individual proteins compared after EVLP were again examined after transplantation (Figures 7, 8 and Table 2). Between the treated and non-treated groups, decreases in proteins involved in inflammation were observed, such as in IL-16 [log(2)-fold change −0.28, q-value 0.55], IL1-RAP [log(2)-fold change −0.68, q-value 0.28], and TLR2 [log(2)-fold change −0.15, q-value 0.89] with values trending toward baseline values (Figure 7). In addition, there were decreases in the same neutrophil-related proteins, such as AZU1 [log(2)-fold change −1.97, q-value 0.16], PRTN3 [log(2)-fold change −2.07, q-value 0.11], MPO [log(2)-fold change −1.80, q-value 0.15], and neutrophil-related elastase [ELANE, log(2)-fold change −1.84, q-value 0.15] (Figure 8). There was also an increase, though not statistically significant, in the macrophage-related regulator protein NOSTRIN [log(2)-fold change 0.68, q-value 0.24] (Figure 8).

Discussion

Given the low utilization of donor organs paired with the high demand, the regeneration and subsequent preservation of otherwise discarded lungs are greatly needed. One methodology

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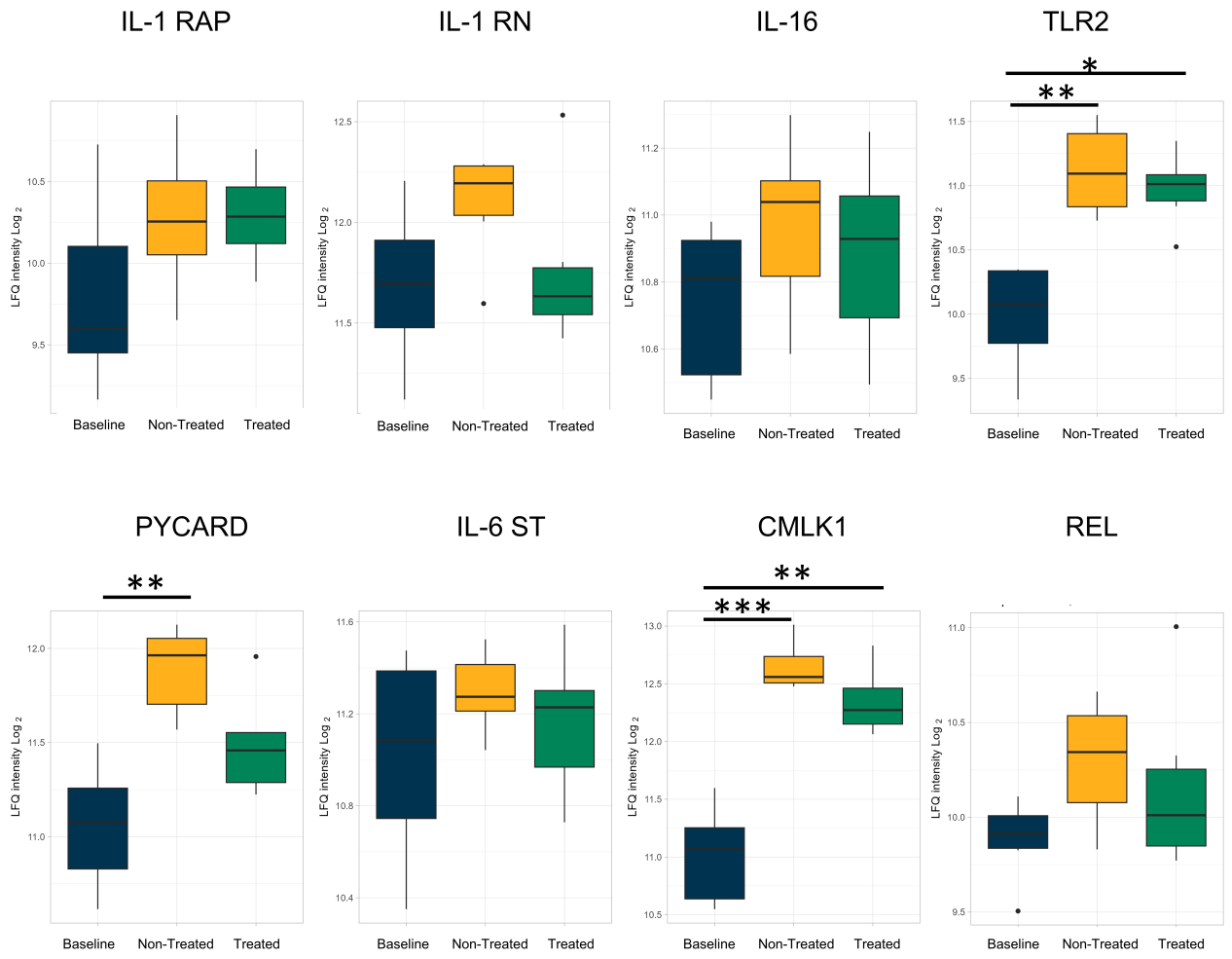

FIGURE 3
Proteins involved in inflammation and related to cytokine processes were upregulated in both the non-treated and treated groups compared to healthy lung (baseline) after EVLP; however, the extent of upregulation was attenuated by the treatment. All graphs represent data from the treated group ($n = 6$), non-treated group ($n = 6$), and baseline group ($n = 6$). The boxplots represent the median and interquartile range (box) with minimum and maximum values (whiskers). Statistically significant differences are reported as FDR-corrected p-values (q-values) using log(2)-fold change differences between groups. IL-1 RAP, interleukin-1 receptor accessory protein; IL-1 RN, interleukin-1 receptor antagonist protein; IL-16, pro-interleukin-16; TLR2, toll-like receptor 2; PYCARD, apoptosis-associated speck-like protein containing a CARD; IL-16 ST, interleukin-16 cytokine family signal transducer protein; CMLK1, chemerin-like receptor 1; REL, REL proto-onco, NK-kB subunit.

for addressing this issue would be treatment with cytokine adsorption, which can be administered during EVLP. However, the biological processes affected by this treatment are not fully characterized. This current study leverages the proteomic analyses performed on lung tissues collected from the lungs treated with cytokine adsorption during EVLP and post-transplantation and non-treated lungs, to better understand this type of graft preservation. The results show that the inflammatory- and immune-related pathways were modulated by the treatment device. The GSEA analysis revealed altered processes related to inflammatory responses, immune responses, stress responses, and leukocyte-mediated immunity pathways. By the end of the observational period after transplantation, the humoral immune response, inflammatory response, and defense response were all significantly downregulated in the treated group compared to those in the non-treated group. This approach of studying treated and non-treated donor lung grafts allowed for a more global understanding of the protein processes affected by cytokine adsorption and showed that the treatment causes broad changes in the inflammatory pathways.

This is important given the existing literature on cytokine adsorption as a treatment that currently focuses on the presence and quantification of particular cytokine levels. In contrast, this study aims to extend our understanding of the treatment effect beyond those parameters to see how larger, more global pathways are affected on a tissue level. As noted, cytokine adsorption has been explored as a treatment modality in the context of EVLP. The adsorber removes the middle- and low-molecular-weight molecules through adsorption to polymer beads, with promising results when applying the adsorber to the treatment of ischemia–reperfusion injury and prolonged EVLP (32–34). For lungs kept in cold ischemia for prolonged periods, such as up to 24 h followed by a longer EVLP duration of 12 h, positive findings of improved compliance and edema have been

TABLE 1 Proteins identified related to the inflammation and cytokine processes compared between the treated and non-treated groups at the end of EVLP.

Protein	Log(2)-fold change	*q*-value
IL-1 RN	−0.27	0.41
IL-16	−0.22	0.24
TLR2	−0.06	0.86
PYCARD	−0.33	0.17
IL-6ST	−0.08	0.83
CMLK1	−0.22	0.42
REL	−0.19	0.57
AZU1	−0.08	0.92
PRTN3	−0.55	0.18
MPO	−0.003	0.99
S100A8	−0.21	0.69
DPP1	−0.28	0.07
ELANE	−0.15	0.76
CD163	−0.48	0.13
NOS	−0.20	0.66
NOSTRIN	0.45	0.07

IL-1 RN, interleukin-1 receptor antagonist protein; IL-16, pro-interleukin-16; TLR2, toll-like receptor 2; PYCARD, apoptosis-associated speck-like protein containing a CARD; IL-6ST, interleukin-6 cytokine family signal transducer; CMLK1, chemerin-like receptor 1; REL, REL proto-onco; AZU1, azurocidin; PRTN3, proteinase 3; MPO, myeloperoxidase; DPP1, dipeptidyl peptidase 1; ELANE, neutrophil-related elastase; NOS, nitric oxide synthase; NOSTRIN, nitric oxide synthase trafficking protein.

demonstrated. The integration of the cytokine adsorber in this case allowed for longer periods of cold storage in the lungs that were healthy at the time of acquisition. In our previous study on cytokine adsorption, the adsorber was used to restore lung function in discarded lungs with signs of ARDS to increase the available donor pool. We showed that the treatment improved pulmonary function during EVLP and post-transplantation, along with a decreased incidence of primary graft dysfunction (19). However, in both of these studies that used adsorption for either longer preservation of healthy lungs or preservation of severely damaged grafts, there has not yet been an exploration of the types of molecular processes affected by the treatment. This current study expands on those translational findings to augment them with a more in-depth proteomic evaluation.

Such work is needed given the number of donor lungs that are routinely discarded. Donor grafts may be rejected due to several etiologies, and ALI due to infection is an important cause. To model this type of infection-induced injury, an LPS-induced ARDS injury was utilized in this porcine model. LPS which is an endotoxin derived from *E. coli* results in endothelial damage that ultimately causes lung injury similar to human pathology. In this study, the resulting ARDS that developed after LPS was given would have resulted in lungs typically declined for use in transplant. Instead, these lungs were harvested and then placed on EVLP. The addition of the cytokine adsorber in line with EVLP allowed for an isolated system in which the lungs could be both treated and evaluated. Subsequently, the lungs were transplanted where they then received further treatment with the cytokine adsorber and were found to have improved function when compared with those in the non-treated group.

Among a small number of studies focusing on cytokine adsorption in the context of EVLP, there are none yet to our knowledge that have investigated the proteomic profiles of the treatment. In this study, proteins were identified using mass spectrometry to characterize the proteome found within the tissues. The resulting differential expression of proteins was computed, and the results showed that each group could be clearly distinguished from one another. Furthermore, the overall differences as shown in the heatmaps both after EVLP and lung transplantation follow-up were significant enough to differentiate the treated group from the non-treated group, as well as each from healthy lung tissue.

Transitioning from the holistic view of the proteome, the biological processes were identified by the GSEA analysis to characterize the biological processes found within the identified proteins. This analysis allowed for the observation of activity patterns across the dataset to see the pathways with biological relevance. From this analysis, the pathways related to inflammatory and immune responses were highlighted, as seen in the GSEA dot plots. In EVLP, the GO terms such as "immune system process" and "coagulation" were further examined particularly given the significance of the terms in relation to ARDS. Post-transplantation, an effect of cytokine adsorption, can be appreciated in the treated group, where biological processes such as humoral immune response, external stimulus response, defense response, and stress response were all suppressed. Cytokine adsorption has been tested in studies for its effect on particular individual cytokines, and these results have introduced the hypothesis that the treatment modality has an overall effect on larger immune responses.

In addition, the biological processes related to coagulation and hemostasis were found to be suppressed in the treated group when compared to the non-treated group at the end of observation after transplantation. This suggests a positive effect on coagulation hemostasis, which would be important in both the setting of the ARDS induced in the donor lung in this experiment and in surgical procedures in general, where the risk of bleeding is an important consideration. This model aims to test whether lungs that had experienced sepsis-induced ARDS could be recovered for lung transplantation using cytokine adsorption, which is significant given the number of grafts declined for use due to infection damage. To this point, coagulation dysfunction is a known sequela of ARDS with activation of the coagulation pathway that is known to perpetuate further damage in lung disease (35). Furthermore, states of inflammation further drive pathological clotting (36). The finding of coagulation hemostasis regulation with the treatment is important, particularly since the observed differences in the coagulation-related gene ontology pathways had a lasting effect since the analysis was performed at the end of the 3-day observation period.

To augment the analysis of pathways, individual proteins were observed to look for patterns across proteins involved in inflammation and immune processes. This included decreases in the treated group that were numeric but not statistically significant in proteins linked to inflammation, such as IL16, TLR2, PYCARD, IL-6ST, CMLK1, and cREL. Pro-interleukin-16

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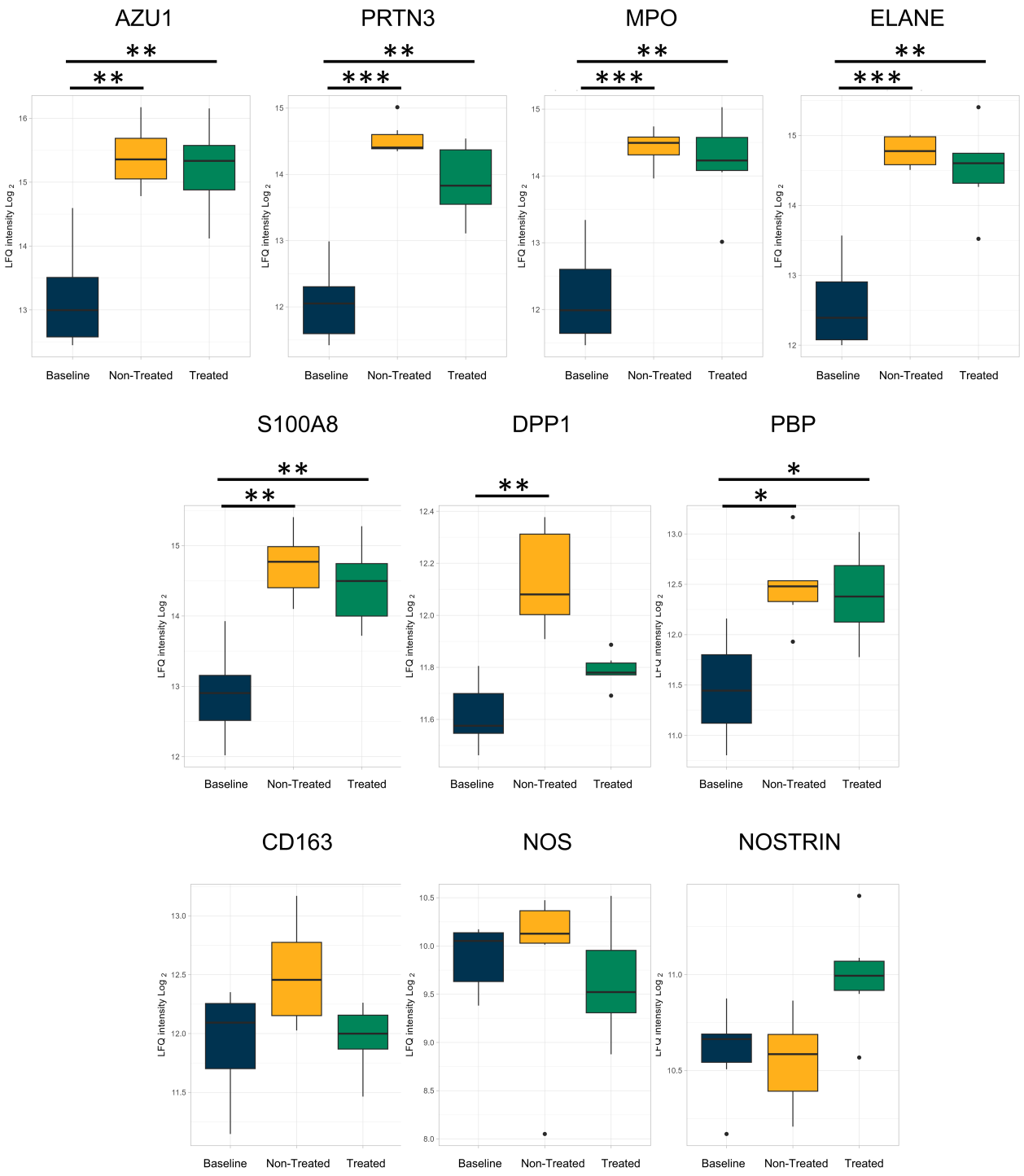

FIGURE 4
Neutrophil- and macrophage-related proteins were less upregulated in the treated group relative to the baseline group compared to the non-treated group as individually analyzed after EVLP. All graphs represent the data from the treated group ($n = 6$), non-treated group ($n = 6$), and baseline group ($n = 6$). The boxplots represent the median and interquartile range (box) with minimum and maximum values (whiskers). Statistically significant differences are reported as FDR-corrected p-values (q-values) using log(2)-fold change differences between. groups Azu1, azurocidin; PRTN3, proteinase 3; MPO, myeloperoxidase; ELANE, elastase, neutrophil expressed; DPP1, dipeptidyl peptidase 1; PBP, platelet basic protein; NOS, nitric oxide synthase; NOSTRIN, nitric oxide synthase trafficking.

served as the precursor to interleukin-16, a pro-inflammatory cytokine that is a chemoattractant and has a direct correlation with the number of infiltrating $CD4^+$ T cells (37). Other cytokine-related proteins identified within the study were related to interleukin-1 and interleukin-6. The interleukin-6 cytokine family is defined by their signal transduction through IL-6ST, which was decreased in this study (38). IL-6 is a significant cytokine given its stimulatory effect on B and T cells and its

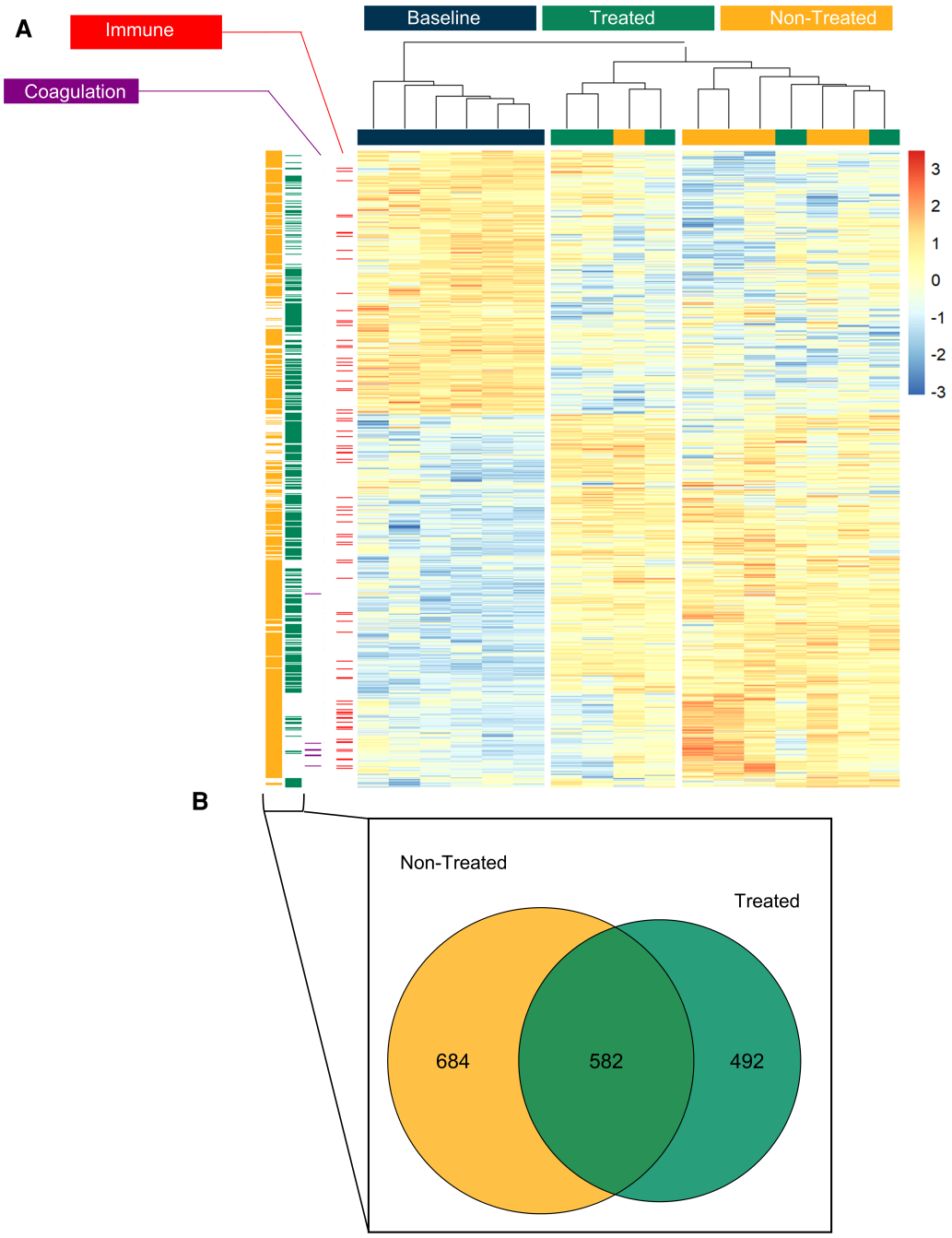

FIGURE 5
The treated group could be clearly differentiated from the non-treated group based on proteomic profiles the end of the post-transplant observational period and both demonstrated a difference from healthy lung tissue (baseline). (**A**) Unsupervised hierarchical clustering was performed on significantly differentially expressed proteins to produce the heatmap across the three groups (baseline, treated and non-treated). Gene set enrichment analysis (GSEA) showed pathways from the biological processes. The proteins from gene ontology (GO) terms are highlighted in columns to the left of the heatmap: the immune system process pathway (red, GO Term GO:0002376) and blood coagulation (purple, GO:0007596). The proteins which were differentially expressed in the non-treated group compared to the baseline are shown separately in the yellow column to the left of the heatmap. The differentially expressed proteins in the treated group are shown in the green column to the left. (**B**) Of those proteins which were differentially expressed between baseline vs treated and baseline vs non-treated, the Venn diagram demonstrates the distribution of unique or shared identities. All graphs represent data from the treated group ($n = 5$), non-treated group ($n = 6$), and baseline group ($n = 6$). Statistically significant differences are reported as FDR corrected p-values (q-values) using log(2)-fold change differences between groups.

described correlation with worsening morbidity and mortality in human ARDS (38–40). Moreover, IL-1 has been specifically identified as a prognostic indicator of outcomes in lung transplant, with the potential to differentiate donor graft performance (41, 42). RNA levels of IL-6 and IL-1 from human donor lungs were also correlated with increased risk of mortality

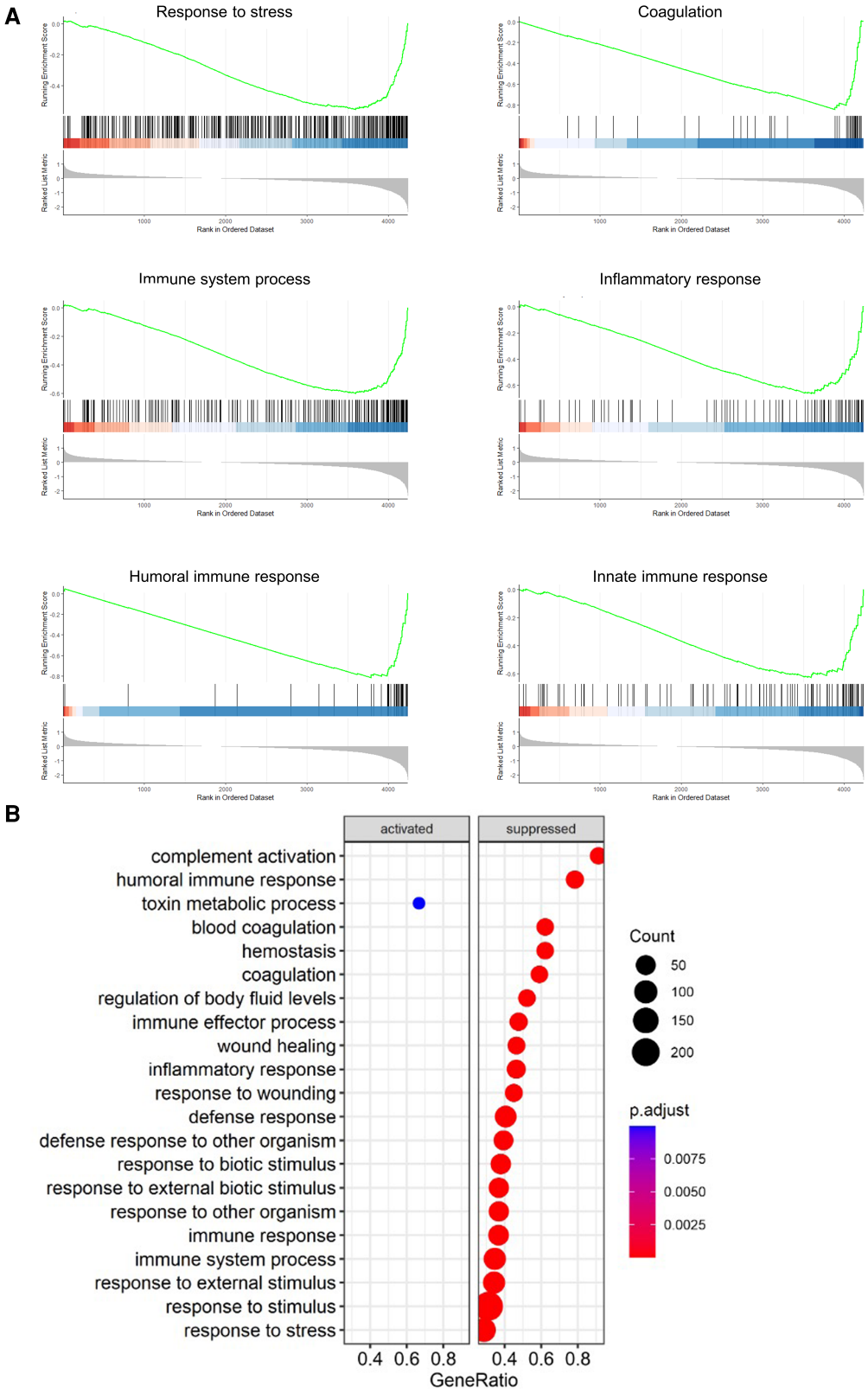

FIGURE 6
Pathways related to immune, inflammatory, and coagulative responses were suppressed in the treated group compared to the non-treated using GSEA after the end of post-transplantation observation. The pathways that were enriched between the non-treated vs. treated comparison are shown as a dot plot. All graphs represent the data from the treated group (n = 5), non-treated group (n = 6), and baseline group (n = 6). Statistically significant differences are reported as FDR-corrected p-values (q-values) using log(2)-fold change differences between groups.

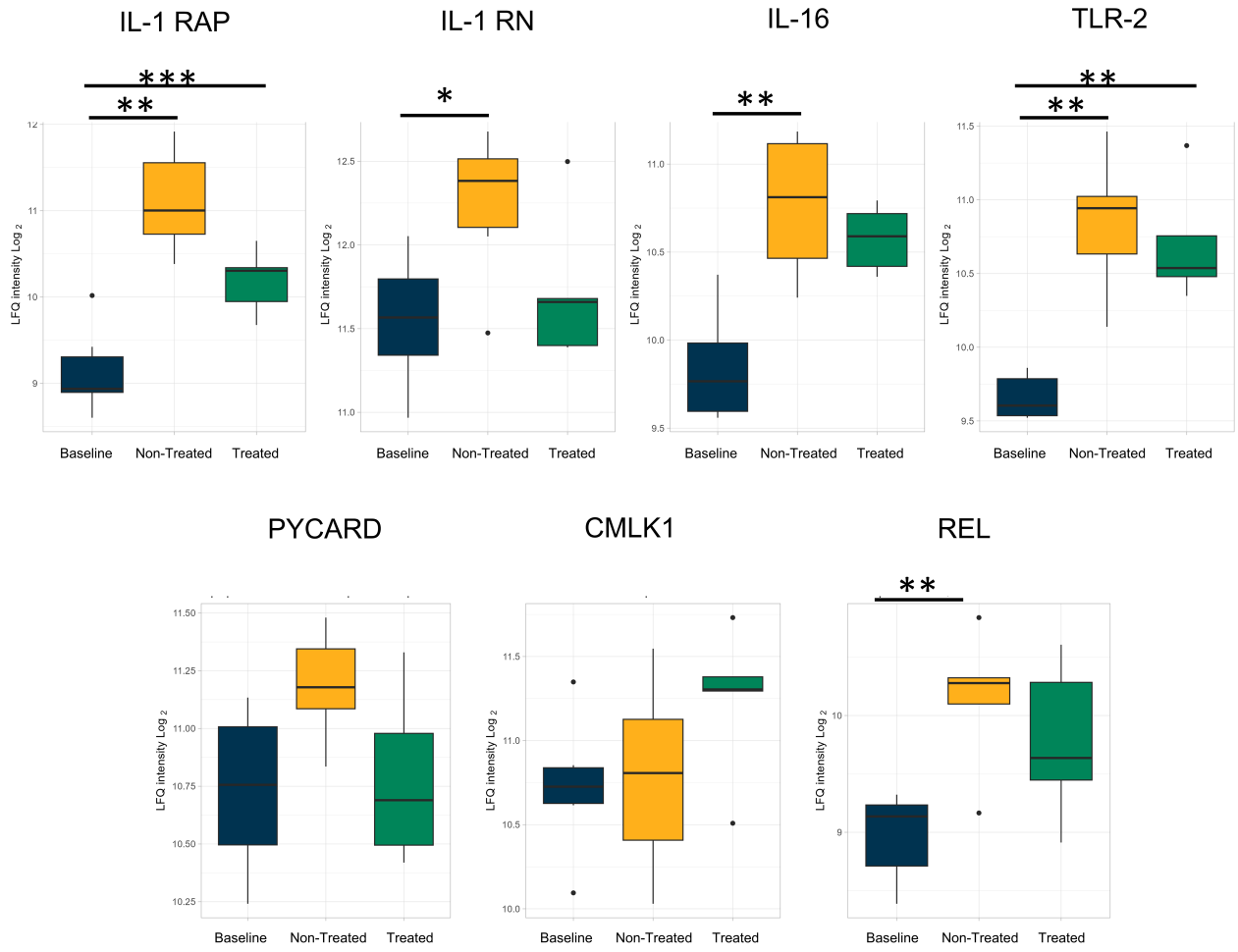

FIGURE 7
By the end of the post-transplantation observational period, the same reduction of proteins involved in inflammation and related to cytokine processes in the treated group was observed after cytokine adsorption in EVLP. All graphs represent the data from the treated group (n = 5), non-treated group (n = 6), and baseline group (n = 6). The boxplots represent the median and interquartile range (box) with minimum and maximum values (whiskers). Statistically significant differences are reported as FDR-corrected p-values (q-values) using log(2)-fold change differences between groups. IL-1 RAP, interleukin-1 receptor accessory protein; IL-1 RN, interleukin-1 receptor antagonist protein; IL-16, pro-interleukin-16; TLR2, toll-like receptor 2; PYCARD, apoptosis-associated speck-like protein containing a CARD; IL-16 ST, interleukin-16 cytokine family signal transducer protein; CMLK1, chemerin-like receptor 1; REL, REL proto-onco, NK-kB subunit.

post-transplantation, supporting the hypothesis that these cytokines and their related pathways are important in mitigating poor outcomes (43). Furthermore, TLR2 is a cell membrane receptor that, similar to other toll-like receptors, recognizes pathogen molecular patterns or pathogen associated molecular pattern (PAMPs) and activates immune cells after PAMP detection. TLR2 has a wide range of PAMP detection including Gram-positive and Gram-negative bacteria and was observed in lower amounts in the treated group in this study (44). The decreases from within the treated group relative to the non-treated group were observed during both EVLP and the post-transplantation period. This points to a sustained effect, particularly given that the cytokine adsorption treatment was given immediately post-transplantation while the lung biopsies were acquired after 3 days of observation.

In terms of proteins related specifically to neutrophils and macrophages, there were several related identities singled out for comparison. Azurocidin, proteinase 3, neutrophil elastase, and myeloperoxidase were detected across the three groups, with modestly lower values in the treated group. These proteins are involved in neutrophil degranulation and neutrophil extracellular traps (45–47). DPP1 plays a role in neutrophil maturation as it activates serine proteinases, and inhibitors of DPP1 have been explored in lung disease as a method of decreasing neutrophil activity (48). The reduction of neutrophilic involvement is a key target in a lung transplantation setting, given the known contribution of neutrophil extracellular traps (NETs) to pathological states with worse post-transplant outcomes (20, 49). In addition, platelet basic protein is a neutrophil chemoattractant and activator, with increasing levels shown to correlate with other forms of lung disease (50).

In proteins related to macrophage function and identity, the macrophage marker CD163 was detected, and a decrease was appreciated between the treated and non-treated groups.

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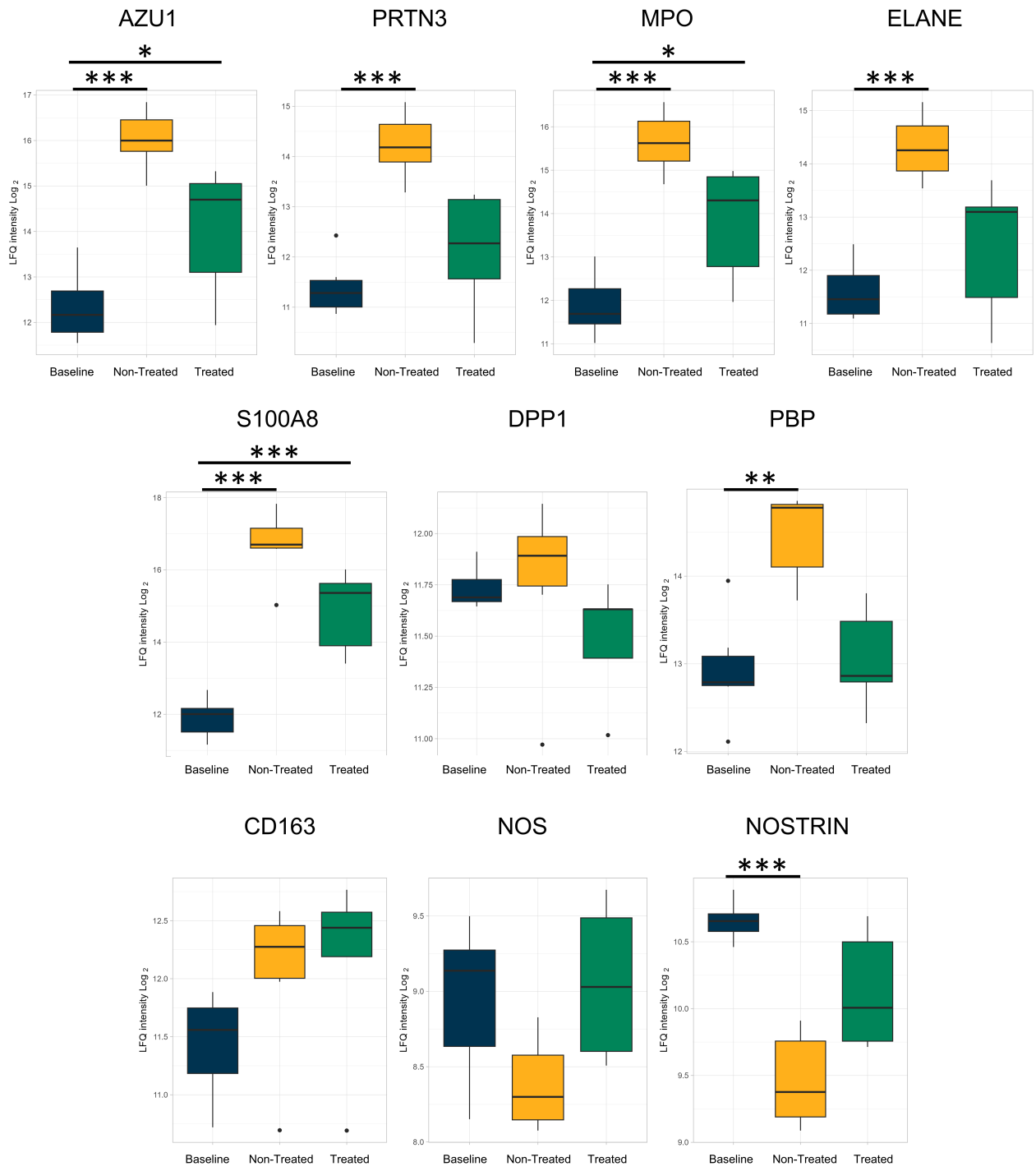

FIGURE 8
Proteins related to neutrophils and macrophage processes that were reduced after *ex vivo* lung perfusion were again reduced in the treated group when compared to the non-treated group after the end of the post-transplantation observation period. All graphs represent the data from the treated group ($n = 5$), non-treated group ($n = 6$), and baseline group ($n = 6$). The boxplots represent the median and interquartile range (box) with minimum and maximum values (whiskers). Statistically significant differences are reported as FDR-corrected p-values (q-values) using log(2)-fold change differences between groups. Azu1, azurocidin; PRTN3, proteinase 3; MPO, myeloperoxidase; ELANE, elastase, neutrophil expressed; DPP1, dipeptidyl peptidase 1; PBP, platelet basic protein; NOS, nitric oxide synthase; NOSTRIN, nitric oxide synthase trafficking.

Moreover, nitric oxide synthase catalyzes the production of nitric oxide needed by macrophages for their oxidative burst. On the other hand, the NOSTRIN is a regulator of nitric oxide, resulting in attenuation of its production through sequestration in endothelial cells and inhibited adhesion of macrophages (51). In this study, NOSTRIN was observed at higher values in the treated group compared to the baseline and non-treated groups, both at the end of EVLP and post-transplantation, which implies

TABLE 2 Proteins identified related to the inflammation and cytokine processes compared between the treated and non-treated group at the end of observation after left lung transplantation.

Protein	Log(2)-fold change	*q*-value
IL-1RAP	−0.68	0.28
IL-16	−0.28	0.55
TLR2	−0.15	0.89
AZU1	−1.97	0.16
PRTN3	−2.08	0.11
MPO	−1.80	0.15
ELANE	−1.84	0.15
NOSTRIN	0.68	0.24

IL-1 RAP, interleukin-1 receptor accessory protein; IL-16, pro-interleukin-16; TLR2, toll-like receptor 2; Azu1, azurocidin; PRTN3, proteinase 3; MPO, myeloperoxidase; ELANE, elastase, neutrophil expressed; NOSTRIN, nitric oxide synthase trafficking.

the presence of different angles from which macrophage involvement is regulated with the treatment. Collectively, the pattern observed both after EVLP and post-transplantation demonstrates that neutrophils and macrophages are affected by the cytokine adsorption treatment. Neutrophils are known to be mediators of inflammation, with NETs contributing to the escalation of inflammatory responses, thrombogenesis, and damage to lung tissue, which manifests as primary graft dysfunction in lung transplantation (49). In addition, the regulation of macrophages is an important process to control within lung transplantation, given the known associations with escalating inflammation and donor macrophages to worsening reperfusion injury (52–54). The finding of reduced markers and effector proteins from within these two immune cell types in the treated group points to an effect of the cytokine adsorber that implies a broader effect. The reduction of cytokines has been explored in other studies when using the cytokine adsorber; however, this study demonstrates that the consequences of the treatment can be more expansive on the immune system and its associated pathways.

The extended effect of the cytokine adsorption treatment can be appreciated when looking at the proteomic changes from the individual level up through the biological processes and then beyond when seeing the changes in overall protein expression profiles within the identified proteome. We observed first that there were global changes that clearly distinguished the treated group from the non-treated group and that further distinguished the treated group from the group with healthy lungs, both after treatment during EVLP and post-transplantation. Furthermore, immune, inflammatory, and defense processes were observed to change throughout the experimental timeline, and the treatment had a further effect on the impact of coagulation, particularly seen post-transplantation. This demonstrates the important effects of the therapy on processes that extend beyond the examination of individual cytokine levels. As cytokine adsorption emerges as a promising therapy for the recovery of marginal and discarded lungs in transplantation, an understanding of the processes that underlie its efficacy is important. We demonstrate here the overarching effects that show that the treatment modulates the immune, inflammatory, and coagulation pathways to change the response in discarded lungs. The findings of this study augment the clinical and histopathological improvements previously seen within studies on cytokine adsorption in line with EVLP and demonstrate the efficacy of using the treatment in graft preservation.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

Ethics statement

The animal study was approved by the Lund University Ethics Committee for Animal Research. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AN: Data curation, Formal Analysis, Investigation, Methodology, Writing – Original draft, Writing – Review & editing, Visualization. GH: Data curation, Investigation, Methodology, Visualization, Writing – Original draft, Writing – Review & editing. LP: Investigation, Methodology, Writing – Review & editing. HG: Investigation, Methodology, Writing – Review & editing. MS: Investigation, Methodology, Writing – Review & editing. SH: Investigation, Methodology, Writing – Review & editing. FO: Formal Analysis, Investigation, Methodology, Writing – Review & editing, Supervision. SK: Data curation, Funding acquisition, Investigation, Writing – Review & editing. SL: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – Original draft, Writing – Review & editing, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Pushing the boundaries of innovation: the potential of *ex vivo* organ perfusion from an interdisciplinary point of view

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Ex vivo machine perfusion (EVMP) is an emerging technique for preserving explanted solid organs with primary application in allogeneic organ transplantation. EVMP has been established as an alternative to the standard of care static-cold preservation, allowing for prolonged preservation and real-time monitoring of organ quality while reducing/preventing ischemia–reperfusion injury. Moreover, it has paved the way to involve expanded criteria donors, e.g., after circulatory death, thus expanding the donor organ pool. Ongoing improvements in EVMP protocols, especially expanding the duration of preservation, paved the way for its broader application, in particular for reconditioning and modification of diseased organs and tumor and infection therapies and regenerative approaches. Moreover, implementing EVMP for *in vivo*-like preclinical studies improving disease modeling raises significant interest, while providing an ideal interface for bioengineering and genetic manipulation. These approaches can be applied not only in an allogeneic and xenogeneic transplant setting but also in an autologous setting, where patients can be on temporary organ support while the diseased organs are treated *ex vivo*, followed by reimplantation of the cured organ. This review provides a comprehensive overview of the differences and similarities in abdominal (kidney and liver) and thoracic (lung and heart) EVMP, focusing on the organ-specific components and preservation techniques, specifically on the composition of perfusion solutions and their supplements and perfusion temperatures and flow conditions. Novel treatment opportunities beyond organ transplantation and limitations of abdominal and thoracic EVMP are delineated to identify complementary interdisciplinary approaches for the application and development of this technique.

KEYWORDS

transplantation, transplantation heart, *ex vivo* organ perfusion, machine perfusion, *ex vivo* machine perfusion, organ modification, *ex vivo* surgery

1. Introduction

The initial idea of isolated organ perfusion was first described in 1812. However, the potential clinical use was only investigated in detail after the introduction of solid organ transplantation to reduce ischemia–reperfusion injury (IRI) caused by the gold standard static-cold organ preservation (SCP). Nowadays, *ex vivo* machine perfusion (EVMP) enables organ perfusion with nutrition- and oxygen-enriched perfusion solutions in hypothermic to normothermic temperature conditions. These protocols led to significantly prolonged preservation times allowing for extended evaluation and potential reconditioning prior to transplantation. Moreover, EVMP dampened IRI associated with a reduction of alloimmune responses (1) (**Figure 1**). Several (pre-) clinical studies in abdominal and thoracic organ transplantation confirmed the significant benefits of EVMP over SCP, finally improving graft function and outcomes after transplantation (2–4). In addition, EVMP application resulted in significantly higher graft utilization rates due to an expansion of the procurement area by enabling long-distance organ transport (5) and the improvement of otherwise non-utilized marginal donor organs, particularly those retrieved from donors after circulatory death (DCD) and extended criteria donors (ECD) (6). These positive experiences paved the way for the continued implementation of EVMP as a daily routine and standard preservation technique for thoracic and abdominal organ transplantation.

In recent years, major efforts have been made to improve preservation strategies throughout all disciplines, employing

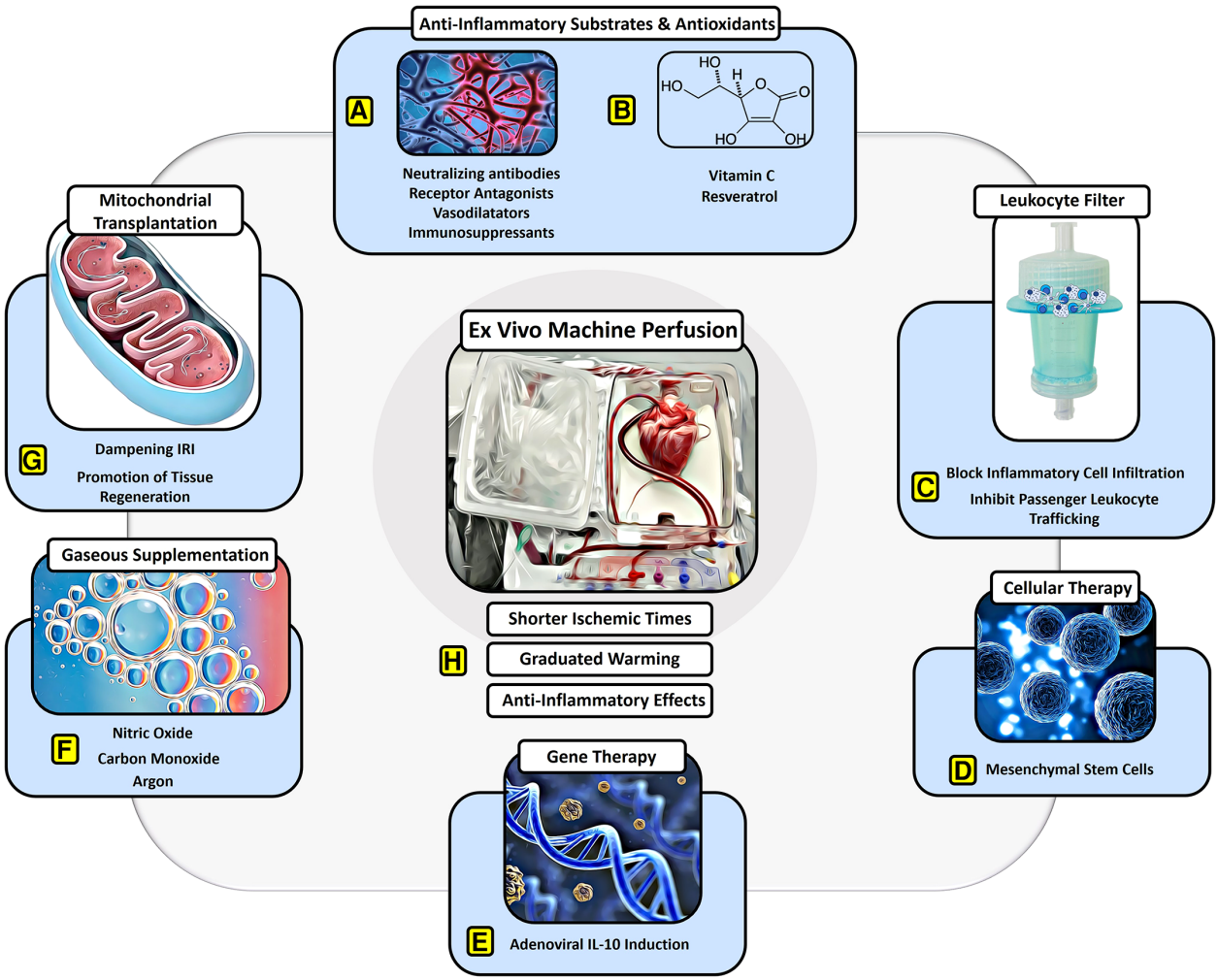

FIGURE 1
Protective effects of EVMP against ischemia–reperfusion injury. EVMP constitutes an interface to administer a broad range of therapeutic components that have focused on dampening IRI. Various (A) anti-inflammatory drugs and (B) antioxidants have been recruited to sufficiently dampen IRI-derived inflammation both experimentally as well as in human studies. (C) Moreover, a leukocyte filter can be recruited to deplete inflammatory cells and inhibit graft infiltration with ambiguous results. (D) Cellular therapies involving mesenchymal stroma cells have delivered promising results on dampening IRI during experimental EVMP. (E) Gene therapies targeting anti-inflammatory pathways such as IL-10 have led to a significant reduction of IRI during lung EVMP. (F) Gaseous supplementation during EVMP aims to promote vasodilation and compromise adverse physical effects on the microcirculation of perfused organs. (G) Mitochondrial transplantation constitutes a novel approach with beneficial effects on IRI in experimental settings for all organs used for transplant. (H) EVMP itself has been delineated to ameliorate IRI by reducing ischemic times, allowing for graduated warming of the organs and perfusion-derived anti-inflammatory effects.

EVMP not only for transplantation but also for a broadened field of applications. These developments focused on the extension of the organ-specific preservation time by optimized preservation protocols, including the improvement of perfusion solutions with the ideal composition of components (e.g., oxygen, nutrition, antibiotics), the perfusion temperature, and flow conditions (e.g., continuous vs. pulsatile) (7). Furthermore, standardized and validated tools for the assessment of organ function during EVMP were established and are currently the subject of investigation to enable the measurement of treatment success and predict post-perfusion outcomes (6). Based on these achievements, EVMP became available for novel treatment concepts, such as the reconditioning and modification of diseased organs *ex vivo*, and for specific tumor and infection therapies during surgical procedures. Furthermore, regenerative approaches in autologous settings are being explored, where patients are on temporary organ support while the diseased organs are being treated *ex vivo*, followed by reimplantation of the cured organ (8–10). In addition, EVMP has emerged as an important tool for preclinical research enabling *in vivo*-like preclinical pharmacological studies with the potential to accelerate the clinical transition of novel therapeutic approaches.

This review provides an interdisciplinary overview of the current abdominal and thoracic EVMP systems and their organ-specific preservation protocols followed by a summary of the relevant EVMP applications beyond organ preservation for allogeneic transplantation. The interdisciplinary application of this new technique may pave the way for researchers to go beyond the boundaries of their own professional discipline, learn from each other, and introduce new ideas in research and clinical practice for the benefit of the patients.

Finally, the authors are aware that a shift of paradigm is currently performed concerning the formerly used term *ex vivo*, which should nowadays only be used when talking about organs from living organisms, e.g., in the case of autologous reimplantation of *ex vivo* cured organs. In the context of allogeneic transplantation, *ex situ* should be used instead, as the organs are recruited from brain-dead or circulatory dead donors. Nevertheless, for better readability, the authors agreed to continuously use *ex vivo* throughout the manuscript.

2. *Ex situ* machine perfusion

2.1. General components and technique

Starting from the simple isolated and research-oriented organ perfusion in the 19th century, the continuous development of medical technology nowadays facilitates the routine application of *ex vivo* perfusion for solid allogeneic organ transplantation. Although a broad range of preservation protocols involving EVMP for different organs have been developed, all *ex vivo* perfusion systems consist of overlapping, general components. These components are combined harmoniously with each other but can be monitored separately to secure the base circulation providing a "near-physiological" condition.

The organ chamber contains the respective organs connected by vascular ports to the perfusion system. The organ is perfused by an integrated centrifugal or roller pump that ensures either a continuous or pulsatile blood flow via the arterial inflow cannula while blood is drained via the venous outflow tract. The downstream reservoir collects the circulating perfusion solution, also facilitating the optical control of a sufficient level of liquid to avoid air aspiration. Through the heater/cooler system, the perfusion solution is constantly tempered hypo-, subnormo-, or rather normotherm, depending on the underlying perfusion protocol. The supplement-enriched perfusate guarantees a nutrition supply, while the oxygenator provides the required oxygenation and decarboxylation of the perfusate as a prerequisite for sufficient cell metabolism. To compromise IRI and alloimmune responses and allow removal of accumulating toxic metabolites during *ex vivo* perfusion, the integration of leukocyte filters has been tested for several organs. However, its efficacy remains unclear as no differences in proinflammatory cytokines and leukocytes or clinical outcome parameters could be observed, probably due to rapid saturation of the filter with donor leukocytes as examined in porcine lung EVMP (11). Although all *ex vivo* perfusion systems operate as closed systems, ports at various locations are incorporated for additive supply and perfusate replacement or sample collection. Finally, a monitoring unit controls not only general (e.g., venoarterial pressures) but also organ-specific features (e.g., heart rate).

2.2. Organ-specific perfusion systems

To fulfill the "near-physiological" environment for the respective *ex vivo* perfused organ, distinct additional components are included in the basic circulation and are realized in various organ-specific *ex vivo* perfusion systems, which are explained below and summarized in **Table 1**.

2.2.1. Kidney systems

In general, a urine reservoir, ports to extract perfusate or urine for assessing graft function, and special cannulas for potential abnormal kidney vasculature are organ-specific components for kidney machine perfusion devices. After the first clinical feasibility study demonstrated safe and promising outcomes in ECD using an EVMP system with a pediatric cardiopulmonary bypass technology (Medtronic) (12), several kidney EVMP systems have been developed, which are currently in clinical use. These involve the LifePort Kidney Transporter (Organ Recovery Systems), Waters RM3 (Waters Medical Systems), Kidney Assist (XVIVO Perfusion), and WAVES System (IGL Group). Although several perfusion solutions are under investigation, the only clinically proven fluid for kidney hypothermic machine perfusion (HMP) is Kidney Perfusion Solution-1 (KPS-1®).

The LifePort Kidney Transporter can be used for both pulsatile and non-pulsatile perfusion at 1°C–5°C and is portable with the possibility of unaccompanied transport, thus reducing logistical efforts and costs. It can be used with any certified machine

TABLE 1 Established EVMP systems for experimental and clinical use for each organ.

Organ	System	Application	Perfusate solution	Perfusion temperature	Pros	Parameters for *ex vivo* monitoring
Kidney	LifePort Kidney Transporter (Organ Recovery Systems)	In clinical and experimental use	KPS-1®	HMP	Lightweight, portable, continuous monitoring, unaccompanied transport, ultrasonic detector to prevent air from entering the vasculature, SCS backup, pressure-controlled pump	Perfusion pressures, renal blood flow, temperature, vascular resistance
	Waters RM3 (Waters Medical Systems)	In clinical and experimental use	Any certified machine perfusion preservation solution eligible for pulsatile flow	HMP	Pulsatile perfusion, dual perfusion possible	Perfusion pressures, renal blood flow, temperature
	Kidney Assist (XVIVO Perfusion)	In clinical and experimental use	Any certified machine perfusion preservation solution	NMP, SNMP, HMP	Pulsatile perfusion, choice of setting the preferred perfusion temperature	Perfusion pressure, renal blood flow, temperature, reservoir temperature, vascular resistance
	WAVES System (Groupe-IGL)	In clinical and experimental use	WATERS IGL® Pulsatile Perfusion Solution	HMP	Lightweight, portable, pulsatile perfusion, unaccompanied transport	Perfusion pressure, renal blood flow, temperature, vascular resistance
Liver	Organ Care System™ Liver (TransMedics, Inc.)	In clinical and experimental use	OCS solution with RBC	NMP	Portable, approved for DBD and DCD livers	Hepatic artery flow (HAF), portal vein flow (PVF), oxygen saturation (SvO_2), hematocrit (HCT), temperature, Hepatic Artery Pressure (HAP), and Portal Vein Pressure (PVP)
	Metra® (OrganOx)	In clinical and experimental use	Any certified perfusion solution compatible with OrganOx guidelines	NMP	Portable, allows perfusion for up to 24 h and a "back-to-base"-mode (NMP following initial SCP)	HAF, PVF, pH, lactate clearance, bile production
	Liver Assist (Organ Assist)	In clinical and experimental use	Any certified machine perfusion preservation solution	NMP HMP/HOPE/D-HOPE	Enables perfusion at every temperature between hypothermic and normothermic, pulsatile arterial and continuous venous flow, automatically adjusts the flow to the natural resistance of the graft	Perfusion time, flow, pressure, temperature, reservoir temperature, vascular resistance Cf-miRNAs from perfusate and bile samples have been used to assess graft viability and function
	Medtronic Portable Bypass System (PBS®)	In clinical and experimental use	Vasosol machine perfusion solution	HMP	Already established in cardiopulmonary bypass and extracorporeal membrane oxygenation	Perfusion time, flow, pressure, temperature, reservoir temperature, vascular resistance
Lung	Organ Care System™ Lung (TransMedics, Inc.)	In clinical and experimental use	OCS solution with RBC	NMP	Portable, potential use for split lung preservation, and *ex vivo* surgery, pulsatile flow, oxygenator can also be used to deoxygenize the perfusate and thus evaluate the oxygenation capacity of the graft	Flow (PF), pressure (PAP), VR, temperature, SaO_2, SvO_2, HCT, PAWP, PEEP, RR, TV
	XPS™ (XVIVO Perfusion)	In clinical and experimental use	STEEN Solution™	NMP	X-ray and CT-scan possibilities, in-line gases with real-time tracking (pO2, pH), separate sterile area and perfusionist non-sterile area	PA and LA pressure, temperature, flow, pH, pCO_2, pump speed
	Lung Assist™ (Organ Assist)	In clinical and experimental use	Any certified machine perfusion preservation solution	NMP	Evaluation in hypothermic and normothermic conditions, compatible with any ventilators, enables perfusion at every temperature between hypothermic and normothermic, portable	Perfusion time, flow, pressure, temperature, reservoir temperature, vascular resistance
Heart	Organ Care System™ Heart (TransMedics, Inc.)	In clinical and experimental use	OCS solution with RBC	NMP	Portable, allows for DCD donations, accepting marginal hearts	Flow (pump, AOF), flow (CF), temperature (temp), pressure (AOP, PAP), heart rate, hematocrit (HCT), saturation (SvO2)
	Heart Box (XVIVO Perfusion)	In clinical and experimental use	XVIVO Perfusion Solution	HMP	Non-ischemic heart preservation (NIHP), used in experimental and clinical xenotransplants	Flow (CF), pressure (AOP, PAP)

perfusion solution eligible for HMP. Investigating the clinical benefits of the system, a reduced risk of delayed graft function, and an improved graft survival in the first posttransplant year when compared to SCS were shown (13). As of today, the LifePort Kidney Transporter is the most used perfusion device for clinical kidney HMP.

The Waters RM3, in turn, is a portable system that provides pulsatile flow for HMP at temperatures between 3°C and 8°C and can be used with any certified perfusion solution eligible for pulsatile flow. In addition to single kidney perfusion, simultaneous perfusion of two explanted kidneys is possible, and the system comes with trident adapters for the cannulation of grafts with anatomical anomalies, such as multiple renal arteries. Experimental dog studies comparing the flow-driven RM3 with the pressure-driven LifePort found no significant differences in transplant outcomes (14).

Another device for kidney EVMP, i.e., the Kidney Assist (XVIVO Perfusion), allows for pulsatile perfusion at a flexible temperature range (12°C–37°C), thus representing the only device capable of kidney normothermic perfusion (NMP). It is FDA-approved and can be used with any certified machine preservation solution, but it is non-portable. Clinical feasibility and safety have been shown with comparable outcomes for both oxygenated and non-oxygenated perfusion (15).

At least, the WAVES System (IGL Group) provides pulsatile HMP (2°C–8°C) using the WATERS IGL® Pulsatile Perfusion Solution. It is portable and designed for unaccompanied transport. Clinical safety has been reported with improved functional outcomes of machine-perfused kidney grafts (16, 17). Of note, it can also be used for combined kidney–pancreas preservation.

2.2.2. Liver systems

Organ-specific modifications of liver perfusion systems include a bile reservoir and a second influx cannula for portal vein perfusion. Currently, three distinct EVMP devices for liver preservation are available for clinical use, namely, Liver Assist (Organ Assist), Organ Care System™ Liver (TransMedics, Inc.), and Medtronic Portable Bypass System (PBS®).

The Liver Assist (XVIVO Perfusions) is the most used EVMP device for liver perfusion that is compatible with any certified machine preservation solution. Providing pulsatile flow at temperatures between 12°C and 37°C, it represents the only currently available device capable of liver HMP or hypothermic oxygenated machine perfusion (HOPE). However, it is non-portable, thus requiring a combinatorial approach with other preservation strategies. Several clinical studies have shown improved transplant outcomes and reduced graft injury in HOPE-treated DCD (18–20) and ECD (21) organs and beneficial effects of NMP and combinatorial approaches (22, 23). The portable OrganOx Metra® allows for extended preservation times of up to 24 h during NMP. The biggest RCT on NMP demonstrated a 50% reduction in discard rates and a 50% lower level of graft injury when compared to CSP (24).

The Organ Care System™ Liver (TransMedics, Inc.) is also portable and uses OCS solution with red blood cells (RBCs) for pulsatile NMP at 34°C. The recent PROTECT trial comparing OCS preservation with SCS found reduced posttransplant allograft dysfunction and biliary complications and an increased use of DCD organs in the OCS group (25). It is FDA-approved for both DCD and DBD donor livers.

The PBS® (Medtronic), originally designed for cardiopulmonary bypass or extracorporeal membrane oxygenation, has also been used for clinical liver HMP (4°C–6°C). It provides pulsatile flow while utilizing Vasosol Organ Perfusion Solution. Clinical studies on the PBS demonstrated a reduction of proinflammatory cytokine production (26). However, there is a lack of recent experimental or clinical data.

2.2.3. Lung systems

For lung EVMP, specific components include a respirator for lung ventilation during machine perfusion, enabling also for different ventilation modes, and an additional port allowing for bronchoscopy. This is based on experimental evidence that has shown the beneficial effects of continuous mechanical ventilation during machine perfusion. In porcine experimental animal models, mechanical airway pressure release ventilation following donation after circulatory death has been shown to reduce lung injury with improved oxygenation and compliance (27) while flow-controlled ventilation preserved alveolar recruitment (28). Moreover, preclinical data show that EVMP can also be used conversely in this setting to deoxygenate the perfusate, thereby assessing the oxygenation capacity of the lungs (29). Other experimental approaches such as airway pressure release ventilation and negative pressure ventilation have also been studied in experimental lung EVMP and were associated with improved pulmonary function (27) and reduced lung injury (30), respectively.

Currently, there are three systems for clinical lung EVMP available including the Organ Care System™ Lung (TransMedics, Inc.), XPS™ (XVIVO Perfusion), and Lung Assist™ (Organ Assist). Although all systems use NMP for graft preservation in clinical settings, they differ in some technical parameters and the utilized perfusate.

The Organ Care System™ Lung uses an OCS solution containing RBCs with an open left atrium (LA) and is the only portable device. It uses pulsatile perfusion for NMP at 34°C–37°C. Clinical safety and non-inferiority compared to SCS have been proven in the INSPIRE trial (5). Moreover, the OCS Lung™ has been evaluated for split lung preservation and *ex vivo* surgery (31). FDA approval has been granted for both standard criteria donor and ECD lung preservation, thereby including both DBD and DCD organs.

The XPS™ system (XVIVO) is non-portable and operates with STEEN™ solution and a closed LA while providing continuous NMP at 35°C–37°C. The NOVEL trial demonstrated the clinical safety and efficacy of the system (32). Of note, the XPS™ has been designed to allow radiographic imaging, thus facilitating x-rays and CT scans of the graft during perfusion (33).

The Lung Assist™ device is compatible with any certified machine preservation solution and operates with the LA being closed. It is also non-portable and provides pulsatile flow. Of additional interest, it allows for isolated in and *ex vivo* perfusion in both hypothermic and normothermic conditions (12°C–37°C). The ventilator is not included in the device, yet any pre-existing ventilator is compatible for use.

2.2.4. Heart systems

In addition to other organs, heart EVMP also demands for organ-specific modifications of the machine perfusion system including cables for defibrillation or pacing. Two machine perfusion systems for the heart are currently in clinical use including the Organ Care System™ Heart (TransMedics, Inc.) and the Heart Box (XVIVO Perfusion).

The Organ Care System™ Heart is portable, uses NMP at 34°C–37°C, and provides pulsatile flow to the graft while being perfused with OCS solution containing RBC and donor blood. In 2015, a non-inferiority study showed non-inferiority compared to SCS preservation and paved the way for clinical application (34). Moreover, clinical studies revealed that the OCS enables heart transplantation from extended criteria DBD (35) and DCD (36, 37) donors. In 2021, FDA approval was granted for organs from DBD donors and, most recently, also for DCD organs, making it the currently only FDA-approved device.

The Heart Box (XVIVO Perfusion), in contrast, uses HMP at a temperature of 8°C and perfuses the heart with an oxygenated cardioplegic nutrition–hormone solution and ABO-compatible packed red cells. It is portable and provides continuous flow. The first-in-human study published demonstrated the feasibility and safety of this preservation technique in clinical heart transplantation (38) and a multicenter clinical trial that started in 2020 (NCT03991923). Of note, the Heart Box has most recently been used in the first xenogeneic pig-to-human xenotransplant (39).

2.3. Perfusion solution

Under (sub)normothermic conditions, perfusion solutions enable the preservation of organs in a pseudo-physiological environment with adequate oxygen, nutrients, and metabolic supply. Perfusates are required to balance cellular hydration and electrolyte homeostasis for edema prevention and also to reduce free radical peroxide scavengers to minimize oxidate injury (40). However, to date, the optimal perfusion characteristics and perfusate compositions for EVMP modes remain undefined and therefore lack standardization. This vagueness is mainly due to the variability of EVMP system application, duration, temperature, and flow conditions. Accordingly, a broad spectrum of solutions with different cellular compositions and additives is available.

2.3.1. Base perfusion solutions

Perfusates are categorized into extracellular (i.e., high-sodium and low-potassium composition) and intracellular (low-sodium and high-potassium formula) solutions. Both variants have been successfully tested in EVMP studies, with their superiority seemingly depending on the case- and organ-specific conditions (41). Of additional importance, the composition of base perfusion solutions may fluctuate depending on the set temperature. Therefore, it is important to ensure correct temperature levels during the entire perfusion process (42). Although the safety and feasibility of extracellular-like Ringer's lactate have been demonstrated in human clinical trials of EVMP (43), the University of Wisconsin (UW) intracellular-type solution also has its raison d'être in organ preservation. When using these solutions clinically, perfusion temperatures were maintained at 34°C and 21°C, respectively (8, 44). More recently, the Institut Georges Lopez (IGL-1) solution emerged as an alternative to UW, featuring lower viscosity and potassium levels and replacing hydroxyethyl starch (HES) with polyethylene glycol (PEG) as an oncotic agent. This solution was used at a temperature of 4°C–6°C (45) (NCT01317342).

The XVIVO Göteborg STEEN Solution is a buffered extracellular solution with well-documented value in the field of lung and liver EVMP and is used at a temperature of 37°C (46). It includes human serum albumin and dextran to provide strong osmotic pressure and coat the endothelium from leucocyte interaction. As such, STEEN perfusion and circuitry have been found to maintain organ stability and functionality—even during prolonged EVMP (46). Notably, this solution can be supplemented with RBCs or remain acellular (41). The armamentarium of perfusates furthermore involves a wide array of modifications, such as the Custodiol-MP histidine–tryptophan–ketoglutarate (HTK) solution with high-flow, low-potassium, and anti-nitrosative/oxidant properties designed for oxygenated EVMP at 4°C (47), the cellular Organ Care System solution with a low-potassium dextran formula (at 37°C) (48), Perfadex as an extracellular and dextran-based electrolyte preservation solution (at 37°C–38°C) (49), or the Celsior solution as a colloid-free extracellular-type solution (at 2°C–8°C) (50). Next to the abovementioned preservation solutions, various others have been described (8, 51, 52).

2.3.2. Cellular and gaseous composition

While hypothermic MP can be conducted with or without active oxygenation, in normothermic MP, adequate oxygenation remains vital and can be delivered either by RBCs, synthetic oxygen carriers, or diffused oxygen by carbogen gas mixtures (53). Since whole blood-based perfusates may exert pathogenic effects deriving from hemolysis and residual blood components including cells, complement, and inflammatory factors (54) while also being associated with logistic hurdles and limited supply (55), leukocyte-/thrombocyte-depleted and plasma-free perfusates have gained popularity in preclinical and clinical studies (12, 56–58).

In most studies, red blood cell-based perfusion solutions have been used. Such perfusates are known to efficiently transport oxygen while their constant flow can mitigate shear stress (59). However, blood-based solutions inherently harbor the risk of infection transmission and transfusion-related incidents including hemolysis. Therefore, a variety of alternatives have been proposed ranging from artificial oxygen carriers such as polymerized bovine hemoglobin-based oxygen carriers and pyridoxylated bovine hemoglobin to acellular oxygen-carrying media such as STEEN (60, 61). These modern solutions also offer the advantage of convenient storage and transport—at similar effectiveness and rheological-hemodynamic characteristics (62, 63).

Uniquely, cell-free perfusates allow for gradual rewarming of the graft from hypothermia to normothermic conditions. This

advantage is significant since the increase in metabolic rate, which is associated with the abrupt restoration of normothermia, is postulated to be a secondary cause of IRI (64, 65). While mixtures with supraphysiological concentrations of oxygen are commonly implemented in EVMP protocols, hyperoxemia and varying oxygen tensions warrant further investigations, particularly when combined with acellular perfusates (41). Interestingly, hydrogen sulfide has been identified as a potential additive to induce a hypometabolic state and reduce oxygen consumption, thereby paving the way for the use of normoxic mixtures (66). Further gaseous supplementation may involve carbon monoxide, which was found to promote vasodilation and reduce IRI (67, 68), or argon, which is currently being investigated (69).

2.3.3. Supplementary substrates

A potpourri of supplementary and modifiable components can be blended into the perfusion solutions, to mimic normal metabolism and recreate a near-physiological milieu. Additives that have been investigated include metabolic substrates, buffers, oncotic agents, anticoagulants, vasodilators, antioxidants, anti-inflammatory molecules, and hormones. Substrates for energy metabolism and nutrients, for example, are essential to perpetuate cellular metabolism during perfusion, thus enhancing cell viability. Additives such as glucose 5% or insulin are popular for all types of EVMP. In addition, pyruvate has been investigated as a metabolic substrate in cardiac EVMP and was found to enhance myocardial metabolism (70). Moreover, buffering agents are essential to maintain near-to physiological pH levels, since variations have been observed to adversely affect other physiological parameters such as pCO2 and $HCO3^{-}$(71). Sodium bicarbonate and calcium gluconate, for example, may serve as universal pH and calcium buffers. Oncotic agents are included in various organ preservation solutions with the rationale of limiting tissue edema and subsequent cell death. Molecules such as HES and PEG have been used and could have further beneficial effects such as mitochondrial and glycocalyx protection (72). In addition, mannitol 10% is a well-established cross-organ applicable ingredient to elevate osmolality (41).

Blood-based perfusates are readily supplemented with anticoagulants to prevent clotting within the tubing lumen and decrease the thrombosis risk. For this purpose, the perfusate is usually heparinized or mesh-filtered (8, 41, 73, 74). Furthermore, nitric oxide (NO) levels are reduced during reperfusion, causing vasoconstriction and ultimately leading to prolonged cellular ischemia and aggravated necrosis (75). For this reason, vasodilators such as verapamil or prostacyclin can be applied to offset the transient vessel constriction upon reperfusion (76). Of note, the value of such medication [i.e., smooth blood (micro) circulation and organ perfusion] in acellular perfusates is yet to be defined (41, 77). Interestingly, in cell-free solutions, the biopolysaccharide dextran has emerged as an essential ingredient, preventing pathological leukocyte–endothelial interaction via antithrombotic properties and protecting the integrity of endothelium-rich organs. Thus, the addition of dextran to the perfusate may contribute to healthy vasculature and stable organ functionality (55). Antioxidants and anti-inflammatory molecules have also been under extensive investigation as supplements since they scavenge reactive oxygen species (ROS) arising from IRI and dampen the immunological response (78). As such, various agents including vitamin C, quercetin, and resveratrol have shown beneficial effects (79, 80). In addition, it is worth mentioning that vitamin C also improves microcirculation and reduces inflammation during EVMP. However, clinical benefits remain controversial (81). Hormones represent another group of potential additives with wide-ranging functional properties (82). In experimental liver NMP, for example, melatonin has been found to prevent oxidative stress and improve vascular conductivity (83). Moreover, dopamine reduced histological signs of damage and improved bile production (84). Other hormones investigated include erythropoietin and glucagon (82, 85, 86). Furthermore, EVMP provides a potential avenue for the administration of therapeutic drugs including chemotherapeutics or antibiotics, antivirals, and antimycotics, to decrease the microbial, bacterial, viral, and fungal load of infected organs and/or in the sense of prophylactic treatment (87). It is worth mentioning that the published protocols reveal a wide variance regarding the additives used. The supplements listed herein, therefore, represent only a selection.

2.4. Perfusion temperature

EVMP techniques can be classified according to the temperature applied during preservation roughly distinguished into cold, subnormothermic, and NMP that have found different implementations in the clinics depending on the organ of interest (**Table 2**).

2.4.1. Hypothermic preservation

Hypothermic preservation at temperatures between 4°C and 10°C allows for the elimination of debris, toxic metabolites, and free radicals produced during hypothermia that would otherwise accumulate during cold static storage (88). First applied in kidney and liver preservation, the hemodynamic stimulation of the graft vasculature was found to compromise endothelial damage while pulsatile flow promoted vascular stress exerting beneficial effects on endothelial gene expression and function. Thus, HMP in both kidney (89) and liver (90) preservation has been found to enhance endothelial NO synthase (eNOS) phosphorylation, thereby preventing vasospasm while promoting NO-dependent vasodilatation at reperfusion.

However, due to the hypothermic state, the metabolic activity of the organ is dramatically impeded during perfusion restricting functional organ assessments (91). More importantly, hypothermically preserved grafts still sustain a cold ischemic injury through the inactivation of Na^{+}/K^{+} pumps (92, 93). In kidney grafts, for instance, functional declines following HMP due to mitochondrial perturbations, decreased cell survival, and endothelial activation have been observed (94, 95). It is noteworthy that marginal donor organs from ECD and DCD donors have been delineated to be even more sensitive to cold

TABLE 2 Different preservation strategies.

	HMP	SNMP	NMP
Temperature (°C)	4–10	20–32	37
Oxygenation	Both	Yes	Yes
Advantages	Elimination of debris, toxic metabolites, and free radicals Reduced endothelial damage, especially with pulsatile perfusion Collection of waste products	Damage from the cold is reduced Possible use as a resuscitation platform Drug administration is possible but inferior to NMP No need for oxygen carriers Collection of waste products	Metabolically active state No damage from the cold Superior for DCD and ECD grafts *Ex situ* graft assessment possible Novel interface for *ex vivo* drug therapies and bioengineering Longer preservation times are possible Collection of waste products
Disadvantages	Damage of the cold Reduced metabolism limits functional assessment Mitochondrial perturbations and endothelial activation Lower compliance in lung HMP Less suitable for DCD and ECD organs *Ex vivo* therapeutic interventions restricted	Metabolic activity is dampened Does not fully protect from reperfusion injury Less investigated	Higher costs and logistical effort High level of ATP depletion Risk of infection

ischemia (96, 97), particularly if the cold ischemic time (CIT) is prolonged (98). Therefore, the utilization of marginal kidneys remains limited while a significant prolongation of HMP preservation times is not feasible (99, 100). Supporting the evidence from kidney HMP, hypothermic preservation during lung EVMP was also associated with impaired metabolism and lower lung compliance (101). The application of *ex vivo* therapies as a promising approach during EVMP is thus restricted due to diminished exposure times of the graft during HMP and compromised pharmacodynamics at low temperatures. This may in contrast also promote the accumulation of the agent with harmful effects following reperfusion.

2.4.2. Subnormothermic perfusion

Subnormothermic machine perfusion (SNMP) involves the preservation of explanted organs at 20°C–32°C and is currently undergoing experimental evaluation. In comparison with HMP, cold-induced graft injury is significantly reduced, while the augmentation of metabolic activity occurring during NMP is dampened at the same time. Thus, a metabolic state requiring additional oxygen carriers for adequate oxygenation is not reached (102). In experimental studies, the beneficial effects of SNMP on DCD grafts have been demonstrated (103). In kidney EVMP, for instance, subnormothermic perfusion at 22°C significantly reduced histological kidney damage and proinflammatory responses (103). Using SNMP in an experimental model of porcine liver EVMP improved endothelial cell function and bile duct injury (104), whereas oxygenated SNMP on human livers preserved liver function with minimal damage and sustained hepatobiliary parameters (44). In a rat DCD model of lung EVMP, subnormothermic perfusion at 28°C was associated with decreased proinflammatory cytokine expression and improved biochemical parameters such as compromised lactate and potassium levels and higher ATP and carbonylated protein levels (29). However, clinical data on the translational relevance of this procedure are sparse, and the Kidney Assist is the only commercially available device for SNMP.

2.4.3. NMP

NMP allows organ tissue to remain metabolically active and precludes exposure to the cold, thus minimizing CITs. Graft preservation thereby enables normal cellular metabolism and recovery of ATP production in almost physiological conditions (58), whereas graft metabolites can be flushed, nutrient supply can be optimized, and microvascular circulation can be maintained. Therefore, NMP is considered the treatment of choice for marginal donor organs with successful clinical studies on the liver, lung, and heart (105–107), including DCD and ECD organs. Moreover, NMP of marginal kidneys has shown beneficial effects in porcine experimental models (108).

Furthermore, NMP is the only form of machine perfusion that enables pretransplant *ex vivo* assessment of the organ that could both alleviate decision-making in graft utilization and allow graft assessment during *ex vivo* therapy (109). Therefore, a broad range of viability criteria, such as lactate clearance, bile production, perfusate pH, glucose metabolism, flow rates, and perfusate transaminases, has been evaluated in liver EVMP (22, 110, 111). However, none of these parameters has been established as clinical guidelines because most studies are invariably based on small series with low case numbers despite being randomized or blinded. Larger collaborative studies that aim to confirm the potential biomarkers or shared databases allowing for the collation of obtained data are necessary and may support clinical translation.

Of further interest, previous studies on heart EVMP demonstrated the feasibility of utilizing solid-phase microextraction (SPME) microprobes with subsequent metabolomic profiling to uncover dynamic metabolic changes associated with organ injury and recovery (112), which may expand the range of parameters monitored during EVMP in future studies.

In addition to functional assessment, NMP constitutes a novel interface for *ex situ* therapies as pharmacokinetics and pharmacodynamics of drugs should not be altered by low temperature during HMP. Most relevantly, NMP has enabled a significant prolongation of preservation times throughout all

organ types with 24 h in kidney (113), 1 week in liver (114), 3 days in lung (115), 24 h in heart (116) EVMP. Prolonged preservation times, in turn, provide the opportunity to perform *ex vivo* therapies that demand long application times including gene therapies and bioengineering and *ex vivo* surgery.

Of note, approaches combining NMP with HMP have also been tested in livers and demonstrated improved functional results (117).

2.5. Flow conditions

Flow conditions are crucial parameters of EVMP as they regulate graft supply with oxygen and nutrients and clearance of CO_2 and metabolic products. In addition, flow conditions have been shown to influence the organ protective effects of perfusion solutions and mediate the occurrence of graft edema (8).

Considering the form of flow, pulsatile and continuous flow applications can be distinguished. Pulsatile flow during cardiopulmonary bypass, for instance, has been found to significantly improve vital organ recovery throughout several types of animal models associated with a preserved microcirculation when compared to continuous flow (118, 119). The pulsatile flow, in turn, generates vascular shear stress, which has been considered to influence endothelial gene expression and function (5, 38). Indeed, pulsatile pressure can enhance renal flow in isolated kidney perfusion, improving vascular conductivity that translates into increased clearance of creatinine, sodium reabsorption, and reduced tubular cell injury (120). Mechanistically, better vascular conductance upon pulsatile perfusion in kidneys could be attributed to improved endothelial release of NO and reduced secretion of endothelin-1 (121). However, studies comparing continuous to pulsatile perfusion in kidney pairs found no significant differences in graft survival and kidney function (7). In addition, an experimental study on porcine lungs reported no significant improvement in lung function parameters upon integration of a modified roller pump generating pulsatile flow (122). Taken together, clinically applicable evidence is scarce, and more research on flow forms is needed, especially considering temperature, perfusate, and the respective organ perfused. Although clinical studies in cardiopulmonary bypass patients indicate beneficial effects of pulsatile perfusion (118), it remains to be elucidated whether this also applies to clinical EVMP.

In addition to the form of flow application, the flow rate constitutes another important parameter for organ protective perfusion that has been mainly studied in lung EVMP. Several protocols using different percentages of the donor cardiac output or fixed flow rates exist including the Lund protocol (100% of cardiac output) (123), the Toronto protocol (40% of cardiac output) (124), and the OCS protocol (2–2.5 L/min) for lung EVMP (5). Since all studies investigating these protocols compare outcomes to SCS, no direct comparison between protocols can be made. Moreover, differences in study design, lung transplant type, and patient characteristics do not allow for statistically significant comparisons between these protocols (125). Noteworthy, experimental studies have also investigated lower flow rates comparing EVMP flows of 40%–20% in porcine DCD lungs. Intriguingly, improved lung function, reduced edema, and attenuated inflammation after transplant were observed when using flow targets of 20% (126). Supporting clinical evidence derives from studies comparing high-flow cellular to low-flow acellular machine perfusion, demonstrating higher transplant suitability, higher wet-to-dry ratio change, and decreased histological lung injury in the low-flow group (127).

3. Potential of EVMP beyond ordinary graft preservation

With a broad range of EVMP systems being available for both clinical as well as experimental applications, novel treatment concepts for both the allo- and xenogeneic environment are being explored. Of translational interest, significant efforts are also being made to investigate innovative approaches for the autologous setting, where the diseased organ will be treated *ex vivo*, while the patient is subjected to temporary organ support followed by replantation of the cured organ. In the meantime, the patient is subjected to temporary organ support such as hemodialysis for the kidney or the molecular adsorbent recirculating system for liver compensation and extracorporeal membrane oxygenation for mechanical heart–lung support (**Table 1**). Most notably, this procedure precludes complications deriving from disproportional organ size and the detrimental side effects of immunosuppression associated with allo- and xenogeneic transplantation while being timely limited only by the *ex vivo* preservation times.

3.1. Ultima-ratio drug therapies

As EVMP enables isolated *ex vivo* perfusion of the explanted organs, it provides a novel interface to treat them by high-dosage medication without the otherwise significant disadvantage of dose-limiting systemic side effects, resulting in more effective therapeutic success. In this context, EVMP has been established as a therapeutic platform to administer ultima-ratio therapies of failing organs in patients with otherwise poor prognoses and non-tolerable contraindications for systemic administration.

This is of particular relevance for the lungs, as severe bacterial lung infections are one of the most frequent reasons for hospital mortality due to sepsis and systemic organ failure (128). Numerous competing factors in critically ill patients have been characterized to impede effective antimicrobial drug dosing required to eliminate pathogens including increased or decreased renal blood flow, renal and hepatic dysfunction, changing volume of distribution, and initiation of mechanical support devices such as continuous renal replacement therapy or extracorporeal membrane oxygenation (129). Thus, high-dosage antimicrobial agents during EVMP with subsequent re-transplantation could provide an option to achieve augmented in-organ doses of antimicrobial agents while sparing systemic side effects. Strikingly, subjecting explanted lungs infected with incurable,

multidrug-resistant pseudomonas aeruginosa to a high dosage of colistin during EVMP enhanced overall survival in a porcine lung autotransplant model (130). Of note, colistin has been shown to exert tremendous side effects causing renal and neurological toxicity with higher cumulative doses, therefore limiting its effective *in vivo* application (131). Consistent with this study, a high dosage of empiric antimicrobial agents added to the EVMP perfusate of marginal donor lungs caused an effective reduction in microbial burden (132), improving pulmonary lung function with increased oxygenation, better pulmonary compliance, and reduced PVR (133). Moreover, isolating the infected organ from the organism for EVMP treatment concomitantly removes the source of infection restricting septicemia and associated systemic immune responses, which otherwise have been associated with accelerated multi-organ dysfunction, compromised antimicrobial drug efficiency, and death (134, 135). In order to further attenuate infectious organ injury during EVMP, novel cellular therapies involving mesenchymal stem cells (MSCs) are currently evaluated in preclinical and clinical trials. Thus, tracheal instillation of MSCs during EVMP of *E. coli*-injured human lungs increased bacterial clearance and dampened inflammatory infiltration and proinflammatory cytokine production while improving alveolar fluid clearance (136) (**Figure 2A**).

Allowing for isolated, high-dosage therapy without systemic distribution of thrombolytic drugs, EVMP may furthermore display an alternative treatment approach to surgical pulmonary embolectomy in patients with large-scale pulmonary embolism and significant contraindications for fibrinolysis. In support, a recent case report demonstrated the feasibility of thrombolysis during EVMP in a donor lung affected by dispersed embolization with improved paO_2, dynamic compliance, and less pulmonary edema allowing for subsequent, successful transplantation (137) (**Figure 2B**).

High-dose application of therapeutic agents such as antibiotic drugs during EVMP demands biomonitoring tools to assess the tissue concentration of the drug. Therefore, novel methods such as *ex vivo* SPME coupled to liquid chromatography/mass

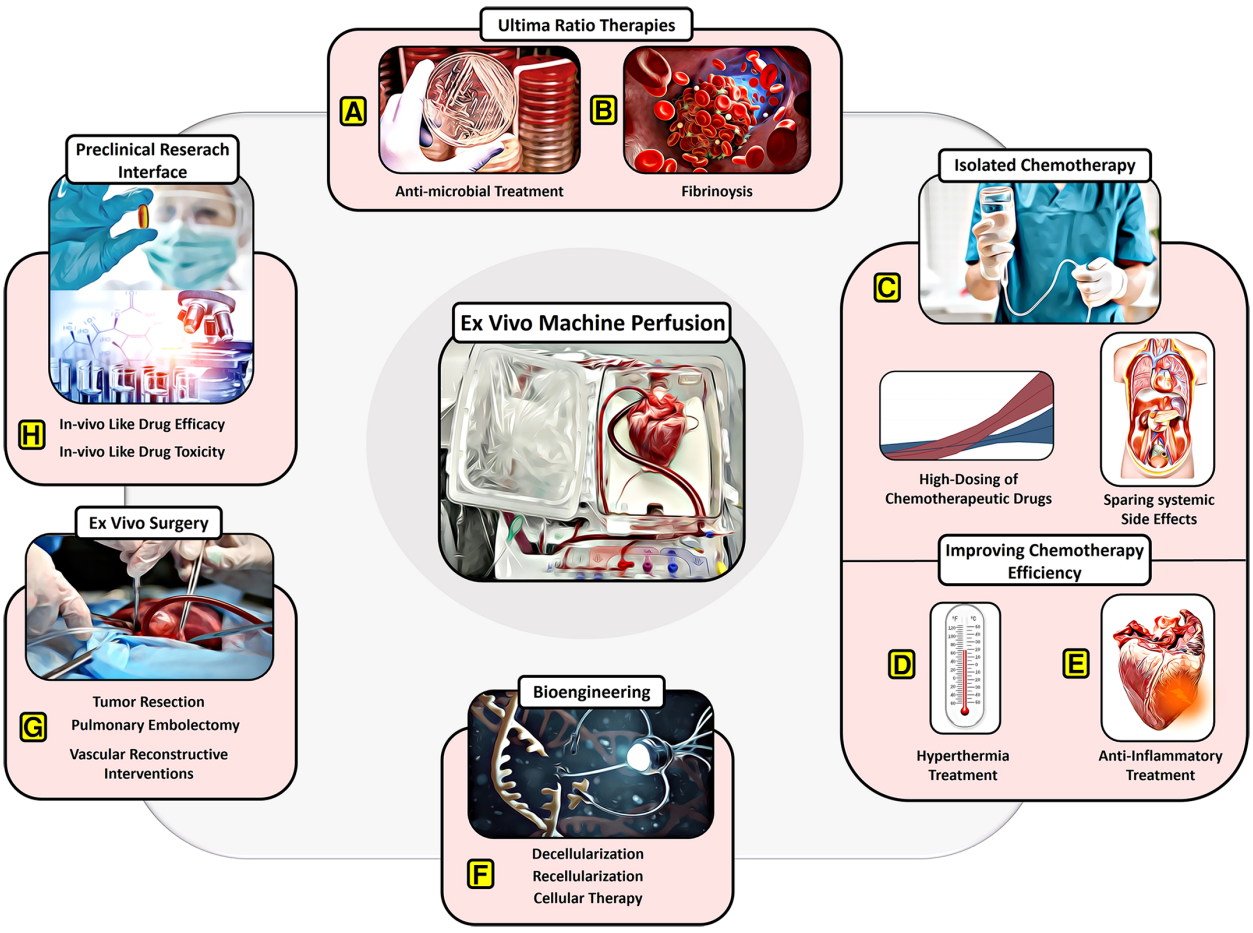

FIGURE 2
Potential of EVMP beyond transplantation. (A) Ultima-ratio therapies involving antimicrobial treatment for severe infections and (B) thrombolytic drugs for severe embolism. (C) Isolated chemotherapy allowing for high dosing of chemotherapeutic drugs despite systemic side effects. (D) Improving chemotherapy efficiency through recruitment of hyperthermic preservation or (E) anti-inflammatory therapies or hypothermic preservation. (F) Bioengineering approaches involving decellularization and recellularization. (G) *Ex vivo* surgery facilitating tumor resection, pulmonary embolectomy, and vascular reconstructive interventions. (H) Providing a preclinical research interface for *in vivo*-like drug testing.

spectrometry allowed for rapid quantification of doxorubicin in porcine lung tissue by inserting a microfiber for 20 min (138).

Considering that many agents with potential for EVMP application display short lifespans, further investigation to define the ideal time point and duration of therapy administration is required. Notably, a recent report demonstrated the utilization of anti-CD31 to enhance the delivery of nanoparticles to explanted human kidney endothelium during EVMP, which can serve as depots for long-term drug release ensuring organ-specific therapy continuation following reimplantation (139). Finally, EVMP could augment the potential of novel gene therapies. Although approved by the FDA within the recent decade, they are associated with a significant economic burden, often requiring multiple applications due to insufficient delivery (140, 141). Here, EVMP could uphold drug levels by isolated target organ perfusion without systemic drug distribution, which would significantly reduce costs and could lead to a more optimized therapeutic effect. Concomitantly, drugs that have shown promising effects, but whose clinical implementation had been hindered due to systemic side effects (142), could find new upwind through EVMP sparing systemic exposure.

3.2. Improved effectiveness of chemotherapy

At this point, EVMP usage focuses exclusively on the autologous setting, where organs will be treated *ex vivo*, while the patients are on temporary organ support, followed by the autologous replantation of the cured organ. In addition to the aforementioned high-dosage application of various medications, chemotherapeutics, hyperthermic conditions, and attenuated local organ inflammation may enhance the effectiveness of the chemotherapy.

3.2.1. Improving chemotherapy efficiency by increased drug doses

Chemotherapeutic drugs display a dose-dependent efficacy on tumor cells in a broad range of cancer types. Thus, platinum-based chemotherapeutics, for instance, that are frequently administered in small-cell lung cancer, have been shown to achieve a significantly higher complete response rate, overall survival, and the number of 2-year survivors when applied at high dosages including cisplatin and carboplatin in contrast to carboplatin alone (143). However, side effects arising from high doses of chemotherapeutic regimens limit their efficacy, thus compromising outcomes of this therapy (144–146). In particular, platin doses are limited by kidney damage arising from acute tubular necrosis and proximal tubule apoptosis (147) and liver toxicity due to hepatocyte necrosis and perisinusoidal fibrosis (148). These systemic side effects could be spared during EVMP, while the lungs could benefit from high-dosage application with significantly improved therapeutic efficiency, thus providing an option for end-stage cancer patients. As a proof of concept, high dosing irinotecan with 20 times higher concentration (2,000 mg/L) than the concentration for systemic application, which engenders strong neutropenia and gastrointestinal toxicity in patients (149), did not induce drug-dependent reperfusion edema or toxic injury to the lung parenchyma (150) (**Figure 2C**).

Moreover, the tumor microenvironment has been shown to exhibit an unbalanced starling mechanism in addition to increased vascular permeability as well as a malfunctional lymphatic system inside the tumor mass, leading to an increased tumor interstitial fluid pressure (TIFP), which in turn impedes drug distribution in many tumors. A significant increase in the composition of the extracellular matrix furthermore compromises drug delivery. Administering additional drugs to the perfusion solution of EVMP to lower the TIFP or augment convection may allow an additional improvement of chemotherapeutic efficacy. Indeed, the angiotensin inhibitor losartan has been shown to reduce stromal collagen and hyaluronan production, therefore reducing TIFP and resulting in increased vascular perfusion potentiating chemotherapy (151). Moreover, blocking the VEGF receptor type 3 was found to decrease lymphatic drainage, thus compromising TIFP and consequent drug removal (152, 153).

3.2.2. Improving chemotherapy efficiency by hyperthermic conditions

A complete explantation of the target organ with subsequent implantation into an EVMP system appears detrimental; however, unique features of modern EVMP systems may improve adjuvant therapies due to the opportunity to influence preservation parameters and the application of therapeutic agents to the perfusate solution. In particular, EVMP enables fine-tuned regulation of the perfusate temperature that could exert beneficial effects during cancer therapy. Indeed, hyperthermia has shown significant antitumor effects by affecting tumor growth directly and improving chemotherapy efficacy (42). Thus, hyperthermic treatment of cancer cells has been shown to induce the DNA damage response by promoting DNA strand breaks, histone g-H2AX foci formation, and ATM phosphorylation, while decelerating DNA replication and repair through downregulated DNA polymerase and topoisomerase activity (154). In the clinics, hyperthermia is already frequently combined with chemotherapeutic regimens leading to higher fluidity of the phospholipid bilayer in tumor cells, thus augmenting drug permeability. Consequently, cisplatin has been shown to exhibit synergistic effects with hyperthermia at 43°C on cell growth inhibition (155). During EVMP, hyperthermia can be easily induced and applied locally targeted to the preserved organ facilitating synergistic cancer treatment with chemotherapeutic drugs and hyperthermia. Further supporting this concept, local hyperthermia in addition to neoadjuvant chemotherapy augmented the effect of the etoposide, ifosfamide, and doxorubicin regimen on soft tissue sarcoma with higher treatment response rate, compromised local progression, and overall improved survival (156). In addition, regional inductive hyperthermia in patients with liver metastasis deriving from breast cancer increased the overall treatment efficacy with a 33.9% higher regression rate (157) (**Figure 2D**).

3.2.3. Improving chemotherapeutic efficiency by dampening local organ inflammation

A broad range of chemotherapeutics including platin, taxanes, 5-FU, and doxorubicin have been delineated to promote a prominent proinflammatory tissue response with the expression of IL-6, IL-8, TNF-α, and INF-β, which in back turn impedes their efficiency and enables metastasis formation (158). Paclitaxel, for instance, induces augmented cytokine production including IL-6, which was mediated via TLR-4 in breast cancer cells. TLR4- expression, in turn, was correlated with conferring resistance to the drug by promoting anti-apoptotic proteins (159), while IL-6 was found to endorse tumor progression inducing angiogenesis and proliferation via the STAT-3 pathway (160). Inhibiting chemotherapy-derived inflammatory signaling has been shown to compromise drug resistance. Neutralizing IL-6 with antibodies, for instance, sensitized multiple tumor types toward distinct chemotherapeutic regimens (161, 162). Since EVMP provides an interface to administer anti-inflammatory agents, utilizing EVMP during *ex vivo* chemotherapy could restrain chemotherapy-derived organ inflammation, thus augmenting therapy efficiency. In support of this approach, the administration of various anti-inflammatory reagents has been tested in clinical EVMP studies showing significant amelioration of donor organ inflammation with compromised inflammatory cytokine expression including IL-6 which ultimately translated into improved function. In lung transplantation, for instance, inhibiting adenosine signaling with A2AR agonists during EVMP inhibited TNF-α, IL-1, and IL-6 expression (163). Similarly, IL-6 receptor blockade with tocilizumab (164), as well as melatonin administration (165) during EVMP, inhibited IL-6-derived IRI in cardiac transplantation. Moreover, in liver transplantation, administration of an anti-inflammatory cocktail comprising alprostadil, n-acetylcysteine, and carbon monoxide in addition to subnormothermic temperature during EVMP restrained TNF-α and IL-6 expression following transplantation (68). At least, the integration of a cytokine filter during lung (166) and kidney perfusion (167) reduced overall proinflammatory cytokine expression with diminished edema formation and improved blood flow, respectively.

In addition to compromising chemotherapy efficiency, the inflammatory response induced by chemotherapeutic drugs furthermore aggravates the function of the target organ. Cisplatin is commonly recruited to treat biliary cancer both in a neoadjuvant as well as palliative setting, thus co-exposing the liver to its toxicity. Notably, platin-based chemotherapeutics have been delineated to promote hepatic injury via oxidative stress and augmented inflammation leading to cellular necrosis (168–170) and organ fibrosis. In contrast, EVMP has been demonstrated to reduce oxidative stress and inflammation in preserved organs, which may in turn dampen chemotherapy-derived organ injury. Metabolomic profiling during liver EVMP, for example, revealed increased ATP levels as well as higher NADPH/NADP ratios associated with reduced lactate levels in liver tissue in the kinetics of 3 h of liver perfusion (171), which may also diminish platin therapy-related oxidative stress. Furthermore, administration of enkephalin, an δ-opioid agonist with antioxidative properties, during liver perfusion compromised oxidative stress and prevented mitochondrial dysfunction, resulting in higher ATP and glutathione in addition to lower AST and malondialdehyde levels in a rat liver EVMP model (172) (**Figure 2E**).

3.3. Bioengineering and organ modification

Bioengineering is considered an innovative and promising future approach with the potential to recondition diseased organs (173). Hereby, EVMP is proposed as a promising interface to deliver cellular products exclusively to the target organ. Strikingly, a recent study reported for the first time the successful engraftment of cholangiocyte organoids into the intrahepatic biliary tree during EVMP, while providing proof of concept that these organoids can repair injured bile ducts. In detail, red fluorescent protein (RFP)-expressing cholangiocyte organoids were injected into the terminal branch of the intrahepatic duct of human, ischemic injured livers at the start of EVMP. Subsequently, organoids exhibited expression of key biliary markers (KRT7, KRT1×9, CFTR, GGT) and improved bile production with increased pH and higher volume despite showing no trans-differentiation into other hepatic lineages (174).

In addition to treating diseased organs during EVMP with subsequent reimplantation, bioengineering is furthermore envisioned to enable the recreation of tissue parts for subsequent implantation as an alternative strategy to organ transplantation. Therefore, EVMP has been proposed as an interface for the decellularization and the recellularization of bioartificial organs under physiological conditions with subsequent implantation (175, 176). In general, decellularization was achieved in a broad range of organs during EVMP with preserved organ architecture and ECM components in addition to low levels of DNA and physiological abundance of glycosaminoglycans and chemical and mechanical components of the ECMs (177). Moreover, administration of human placenta-derived endothelial progenitor cells (EPCs) during EVMP was shown to induce successful recellularization with proliferative EPCs repopulating kidneys, lungs, and hind limb vascular intimae. Of note, a vascular chimerism with human EPCs lining the luminal surface of rat blood vessels, alongside rat cells within the tunica media and beyond, artificially generating vascular chimerism (176). In addition, various studies have demonstrated the effective decellularization of organs from different animals preserving extracellular matrix composition and architecture to engineer bioartificial organs via repopulation with human cell lines (173). Notably, infusing human endothelial cells *ex vivo* through the renal artery and vein of decellularized rat kidneys resulted in homogenous distribution in the vasculature compartments with site-specific endothelial specialization (178). Similarly, administering human perivascular and endothelial cells through the pulmonary artery and pulmonary vein in isolated and decellularized rat lungs and

human lung lobes resulted in successful endothelial cell coverage with functional vascular lumen structures being detected (179). Underscoring the clinical feasibility of bioengineering, this study also examined the transplantation of the recellularized lungs into rats showing the formation of continuous, polarized vascular lumens that remained perfusable 3 days after transplantation (179). Similarly, transplanting clinically scaled porcine liver scaffold with human umbilical endothelial cells (HUVECs) revealed HUVECs localization within sinusoidal regions in addition to expression of a liver sinusoidal endothelial cell-like phenotype. Strikingly, subsequent heterotopic transplantation into immunosuppressed porcine recipients resulted in 15 days of continuous *in vivo* perfusion of the revascularized bioengineered liver (rBEL) (180) (**Figure 2F**).

Mitochondrial transplantation constitutes an additional and very novel approach to modify tissue homeostasis and diseased organs, which may be of translational relevance for EVMP-based regenerative therapies (181). Indeed, mitochondrial transfer has been demonstrated to improve IRI in a broad range of organs while also ameliorating pathological tissue dysfunction. Injecting mitochondria into the hearts of diabetic rats following IRI, for instance, resulted in the recovery of left ventricular function and a reduction of infarct size (182). Likewise, mitochondrial transplantation into the spleen improved liver function while administration via pulmonary artery vascular delivery or tracheal aerosol delivery improved lung mechanics and reduced lung tissue damage following IRI (183). Similar effects had been observed when performing mitochondrial transplantation through renal arteries with protective effects against renal IRI (184). Noteworthy, the effects of mitochondrial transplantation on organ reconditioning have also been delineated with isolated mitochondria of HepG2 cells injected into high-fat diet-fed mice, effectively improving non-alcoholic fatty liver disease (185).

Of relevance, most studies have indicated that the therapeutic effects of a single administration of mitochondria may be transient. Therefore, EVMP may allow higher doses and prolonged exposure to mitochondrial-carrying vectors while providing an interface to determine the time point and route of administration as well as the impact of repetitive cycles (186).

Bioengineering approaches using EVMP are also recruited to create immunotolerance to effectively counteract both the lifelong intake of immunosuppressive drugs and the risk of chronic rejection. One approach focused on the genetic modification of the MHC complex and the minor histocompatibility antigens (mHag) of the vascular endothelium. Notably, administration of short hairpin56 RNAs targeting beta-2 microglobulin and class II transactivator transcripts using lentiviral vectors during EVMP resulted in durable MHC I and II complex suppression without affecting cell viability or tissue integrity (187–189). Another approach utilized adenoviral vectors to induce IL-10 expression in donor lungs to prevent the development of primary graft dysfunction in a large animal survival model. Indeed, this approach was shown to be safe, to improve lung function, and to have an immunological advantage in both innate and adaptive immune responses (190).

3.4. *Ex vivo* surgery

Allowing for increasingly prolonged *ex vivo* preservation of organs, EVMP has paved the way for surgical *ex vivo* procedures. Utilizing stained perfusion solutions (e.g., methylene blue) in addition to altering flow conditions hereby allows for visualization and therefore suturing of even smaller leakages avoiding significant blood loss over time. Moreover, enabling the visualization and access to all organ sites and a prolonged period of time for accurate tissue preparation and reconstruction, this technique has raised significant interest throughout all disciplines utilizing EVMP.

For surgical management of renovascular diseases, *ex vivo* surgery is associated with significant technical advantages allowing for vascular reconstructive interventions. Thus, laparoscopic nephrectomy with subsequent autotransplantation was successfully utilized for renal artery aneurysms affecting distal vascular branches (191) and for nephron-sparing resection in the case of a large renal tumor (192). Integration of an EVMP system for *ex vivo* vascular kidney surgery, in turn, was associated with improved assessment of the perfusion characteristics of the remodeled kidney, in a study performing *ex vivo* surgery in patients with a solitary kidney and either dysplastic aneurysm, Takayasu disease, or fibrodysplasia lesions (193), thus preventing nephrectomy and lifelong dialysis.

Liver and intrahepatic bile duct cancers and hepatic metastases deriving from other extrahepatic tumors account for the most prevalent tumors in humans (194). Surgical resection hereby displays the gold standard therapy for most of these pathologies. However, compromised liver function and lesions at difficult anatomic sites, for instance, with the involvement of larger vessels constitute a contraindication for curative surgery. Since then, various studies utilizing *ex vivo* surgery to resect complicated hepatic malignancies including hepatocellular carcinoma, cholangiocellular carcinoma, and focal nodular hyperplasia and hepatic metastasis, achieving significant R0 resection rates (93,4%, CI: 81.0%–97.9%). However, these interventions had been associated with high 30-day mortality (9.5% CI: 5.9%–14.9%) (195). Integrating EVMP during *ex vivo* surgery could enable a prolonged preparation and aftercare during the operation in addition to ameliorated IRI upon reimplantation. Moreover, recruiting hyperthermia and *ex vivo* chemotherapy during surgery may further adjuvate tumor elimination. Of note, *ex vivo* liver resection has also been introduced as a curative approach for non-resectable, end-stage hepatic alveolar echinococcosis (HAE) associated with dissemination into the intrahepatic conduits and adjacent structures. Thus, a recent study reported a curative treatment in 29 of 31 patients with long-term recovery and no HAE recurrence (196).

The feasibility of EVMP-supported operations in thoracic surgery has been demonstrated in porcine models of large tracheobronchial leakage with successful implantation of a pericardial patch and replacement of the distal trachea with an aortic graft using the OCS (197). Moreover, EVMP has already been applied in the clinical setting with successful lung

autotransplantation for centrally located and locally advanced lung cancer to spare lung parenchyma by avoiding pneumonectomy, which underscores the potential of this procedure (198). Noteworthy, integrating an EVMP system could furthermore enable the application of topical, high-dosage therapeutic drugs avoiding systemic side effects, which is of particular interest in supporting the long-lasting success of *ex vivo* tumor resections in lungs (199). Moreover, surgical procedures can be performed in the absence of ventilation improving surgical accuracy, which otherwise is hardly possible during in-human surgery, in particular in patients with threatening decreased lung function.

Exploiting the improved access routes to critically located tumors, various case studies in cardiac surgery have reported successful *ex vivo* resections of cardiac sarcoma (200), complex atrial myofibroblastic tumors (201), and giant large atria following chronic mitral valve disease on an *ex vivo* beating heart (202). Moreover, single centers also have reported on larger case numbers of cardiac autotransplantation with *ex vivo* tumor resection for malignant complex primary left heart tumors (203) (**Figure 2G**).

3.5. Preclinical in-human research

Being able to preserve solid organs in a perfused, physiological environment enables a new field for *in vivo*-like preclinical studies with the potential to accelerate the clinical transition of novel therapeutic approaches. Therefore, a large choice of animal models for kidney, liver, and thoracic organ EVMP have been developed, which simultaneously facilitate the investigation of therapeutic regimens for diverse disease models. However, multiple differences, for example, between rodent and human perfusion models restrict the translational relevance of these studies due to lower EVMP perfusion flow in rat models (204) and hypersensitivity toward dextran-based perfusion solutions, which does not occur in humans (205). Moreover, a broad range of diseases, in particular various malign tumor animal models, are still lacking substantial transferability with regard to the strong heterogeneity of tumorigenesis (206).

Utilizing organs from deceased patients in contrast enables the opportunity to test novel pharmacotherapeutic therapies in relevant human disease models and allows for more precise prediction of therapy efficacy when compared to animal models.

For lungs, acute respiratory distress syndrome (ARDS), for instance, represents an acute life-threatening pathology frequently deriving from severe infection, which evolves rapidly and confers high mortality on the afflicted (207). Advances in clinical care have significantly improved ARDS outcomes (208); however, no appropriate pharmacotherapy has emerged. A wide range of anti-inflammatory agents (e.g., corticosteroids, prostaglandins, n-acetylcysteine) had provided promising results in both rodent and large animal models, however without translational relevance, as clinical trials failed to achieve significant benefit for patients (209). In contrast, a recent study applied EVMP in human donor lungs, which were not acceptable for transplantation, to successfully establish a model of endotoxemia-derived lung injury via lipopolysaccharide (LPS) instillation into the pulmonary artery (210). Tightly reflecting the clinical setting, this approach resulted in a robust cytokine response, along with decreased pulmonary venous oxygen content over five hours as seen during ARDS. Supporting the concept of early pharmacological studies using EVMP, evaluating a novel small molecule, BC1215, which suppresses NF-κB signaling, in the ARDS model resulted in reduced induction of IL-1, IL-6, and IL-10 as measured by ELISA of BALF. RNA sequencing furthermore revealed that BC1215 administration after LPS exposure significantly blunted the NF-κB transcriptional response and preserved venous partial oxygen pressure (210).

In addition to evaluating the efficiency of pharmacotherapies, EVMP could furthermore support clinical phase 1 and 2 studies allowing for the delineation of organ-specific side effects, which are of crucial relevance for clinical transition. Commonly used animal models to test the hepatotoxicity of novel drugs, for example, display significant discrepancies from the human setting due to differences in drug metabolism and mostly homogeneous environmental and genetic conditions in inbred animal strains. In contrast, an EVMP model investigating the impact of acetaminophen-induced liver injury utilized human liver tissue from partial liver resections to mimic the clinical setting. Notably, the EVMP system allowed for hourly perfusate sampling and live assessment of clinical parameters showing compromised liver function during APAP poisoning with lower glucose consumption and lactate production rates while hepatocyte synthesis capacity had been preserved (211). Of note, evaluating liver function by clearance of indocyanine green revealed stable hepatocellular function during the entire perfusion period indicating a clinically relevant setting (211).

Finally, the utilization of EVMP models to initiate clinical trials could lower the ethical burdens of testing novel pharmacological drugs, thus accelerating the transition of promising candidates into the clinics (**Figure 1H**).

4. Outlook and limitations

EVMP—primarily applied in allogeneic organ transplantation—concomitantly provides an interface to investigate perfused organs in an almost physiological setting but with improved accessibility, which was the basic requirement to transfer and test out this technique in a broader spectrum. Applying *ex vivo* therapies to regenerate or cure diseased organs constitutes a feasible approach, which will be further expanded in the future for selected clinical indications thus having the potential to minimize the gap between demand and supply in organ transplantation. This can be implemented on the one hand by the improvement of otherwise discarded, marginal donor organs, in particular from donors after DCD and those retrieved from ECD (6). On the other hand, treating diseased organs *ex vivo* followed by autologous replantation of the cured organ may reduce the number of patients in need of organ transplantation (8–10). This autologous application should be limited to clinics that display profound experience in the field of temporary organ

support systems and organ transplantation. It is noteworthy that, at the current state, the broad majority of studies investigating *ex vivo* supported organ reconditioning are still executed on a purely experimental level and derive from small study series.

Overall, there are two main factors to further advance this innovative research field in the future. At first, further improvements in preservation protocols enabling >24 h *ex vivo* perfusion or even more without significant organ damage need to be established. On the other hand, interdisciplinary exchange and cooperation need to look beyond the boundaries of the own professional discipline to learn and pick up ideas from related ones. The translation into the own clinical or research-associated area may enhance the welfare of patients, true to the saying "You dońt have to reinvent the wheel."

Author contributions

JI: Writing – original draft. AS: Writing – original draft. SK: Writing – review & editing. TN: Writing – review & editing. LW: Writing – review & editing. MR: Writing – review & editing. FH: Writing – review & editing. AN: Writing – review & editing. CKu: Writing – review & editing. FI: Writing – review & editing. VF: Writing – review & editing. MS: Writing – review & editing. AR: Writing – review & editing. AH: Writing – review & editing. CKn: Writing – review & editing. ST: Writing – review & editing. FV: Writing – review & editing. BW: Writing – review & editing.

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Conflict of interest

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Gene therapy during *ex situ* heart perfusion: a new frontier in cardiac regenerative medicine?

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Ex situ organ preservation by machine perfusion can improve preservation of organs for transplantation. Furthermore, machine perfusion opens up the possibilities for selective immunomodulation, creation of tolerance to ischemia-reperfusion injury and/or correction of a pathogenic genetic defect. The application of gene modifying therapies to treat heart diseases caused by pathogenic mutations during *ex situ* heart perfusion seems promising, especially given the limitations related to delivery of vectors that were encountered during clinical trials using *in vivo* cardiac gene therapy. By isolating the heart in a metabolically and immunologically favorable environment and preventing off-target effects and dilution, it is possible to directly control factors that enhance the success rate of cardiac gene therapy. A literature search of PubMed and Embase databases was performed to identify all relevant studies regarding gene therapy during *ex situ* heart perfusion, aiming to highlight important lessons learned and discuss future clinical prospects of this promising approach.

KEYWORDS

gene therapy, heart transplantation (HTx), heart failure, regenerative medicine, *ex situ* heart perfusion

Introduction

With the introduction of machine perfusion for organ preservation, a new era within transplantation medicine has emerged. While conventional static cold storage is still the most commonly employed method for organ preservation, it is associated with a number of limitations, including tissue damage by prolonged hypothermia, limited possibilities for quality assessment, inevitable ischemia-reperfusion injury upon rewarming and reperfusion, and limited options for organ reconditioning. With the growing use of organs of marginal quality from extended criteria donors, these limitations impair clinical transplantation and contribute to the increasing supply-demand mismatch of donor organs (1, 2). By using machine perfusion, it is possible to overcome these limitations by providing a controlled flow of perfusate with a desired composition at a prespecified

Abbreviations
ESHP, *ex situ* heart perfusion; Ad, adenovirus; AAV, adeno-associated virus; PFU, plaque-forming units.

temperature, thereby facilitating the maintenance of tissue metabolism and removal of waste-products, while also serving as a platform for quality assessment and organ reconditioning (1).

In the context of the heart, the introduction of *ex situ* heart perfusion (ESHP) has increased the pool of available donors. It has done so by facilitating transplantation of hearts from extended-criteria donors, hearts from donors in remote geographical areas, and hearts donated after circulatory death, all with satisfactory clinical outcome (3–9). Following the increasing clinical use of ESHP, one can speculate about other uses for ESHP besides facilitating organ preservation, such as biological modification to improve clinical outcome (10–12). One such approach is cardiac gene therapy. In short, gene therapy can be defined as the delivery of therapeutic genetic material by different carriers (vectors) to cells with the aim of preventing or curing a disease by modification of a critical pathophysiological pathway or correction of a genetic defect (13). Most clinical *in vivo* cardiac gene therapy trials, however, yield overall unsatisfactory outcomes, to an important extent due to inadequate delivery and uptake of the viral vectors and expression of the gene product (14–17). These studies mostly used direct myocardial injection, or (percutaneous) intracoronary infusion as a means of vector delivery. In addition, concerns surrounding systemic side effects limit its applicability. This is perhaps best reflected by very high vector dose requirements for the most frequently used viral vector system, adeno-associated viral (AAV) vectors, that result in substantial (undesired) transduction of other organ systems such as the liver (18), leading to inflammatory stimulation (19) and potential activation of proto-oncogenes or disruption of tumor suppressor genes due to viral vector integration (13, 20–22).

ESHP provides the unique opportunity for local or intracoronary delivery of high concentrations of vectors without significant systemic off-target organ or immunological side effects. By isolating the heart in a metabolically favorable environment during ESHP, higher concentrations of vectors can be administered without systemic side effects. This opens up the possibility to modulate the inflammatory response associated with allotransplantation, thereby reducing the need for immunosuppression in recipients (23); improve tolerance to ischemia-reperfusion injury to reduce the risk of primary graft dysfunction; or excision and selective treatment of the heart to correct a pathogenic genetic defect at an early stage in a known carrier, followed by reimplantation of the heart into the same patient (autotransplantation) (**Figure 1**). The latter would prevent the need for heart transplantation in this subset of patients altogether. Given the fact that genetic causes play a substantial role in the etiology of many cases of heart failure (24–27), gene therapy during ESHP followed by autotransplantation might be especially relevant as a potential treatment option for carriers of known pathogenic mutation, preferably at earlier stages before a clinical phenotype has developed. Furthermore, interventions to treat these pathogenic mutations during ESHP could also be applied during isolated *in situ* loco-regional perfusion of the heart (28), which is currently being developed for clinical application [e.g., DiNAQOR AG (Schlieren, Switzerland)].

The objective of this systematic review is to summarize the available literature regarding the application of gene therapy during ESHP and discuss future clinical prospects based on the evidence found in the literature.

Materials and methods

A literature search of the PubMed and Embase databases was conducted up to the 1st of July 2023. The search string is available in the appendix. Identified articles were uploaded to EndNote (Clarivate Analytics, Philadelphia, USA) for duplicate removal. After duplicate removal, the remaining studies were uploaded to Rayyan.ai for title/abstract screening. Studies were included if gene therapy was investigated during ESHP and ESHP was specifically used as a platform for intervention in small and large animal models. ESHP was defined as any form of coronary perfusion through the aortic root after excision of the heart, including bolus injection. Studies not adhering to this definition of ESHP (for instance selective intracoronary infusion or single flush after aortic cross clamping but before excision) were excluded. Remaining studies were subjected to full-text analysis before inclusion into our review. Reference lists of included articles were searched to identify additional studies. Title/abstract screening and full-text analysis was conducted by two authors independently (MTV, JJGJA). Disagreements were resolved by discussion and, if necessary, consultation of a third researcher not involved with the search and selection process (MM).

Results

A total of 2,462 studies were identified after duplicate removal. After screening and full-text analysis, a total of 23 studies that specifically addressed the application of gene therapy during ESHP were retained. Based on the identified studies, we made a distinction between gene therapy applied during hypothermic ESHP (**Table 1**) and normothermic ESHP (**Table 2**), with hypothermic ESHP being the focus of most papers.

Gene therapy during hypothermic *ex situ* heart perfusion

Most research into gene therapy during hypothermic ESHP involved adenoviral (Ad) mediated gene transfer (29–41). Three studies could be identified that involved AAV (42) or liposome-mediated gene transfer (43, 44). Hearts were mostly harvested from rats (29–33, 36, 37, 39–42, 44) or rabbits (34, 35, 43), one study involved porcine hearts (38).

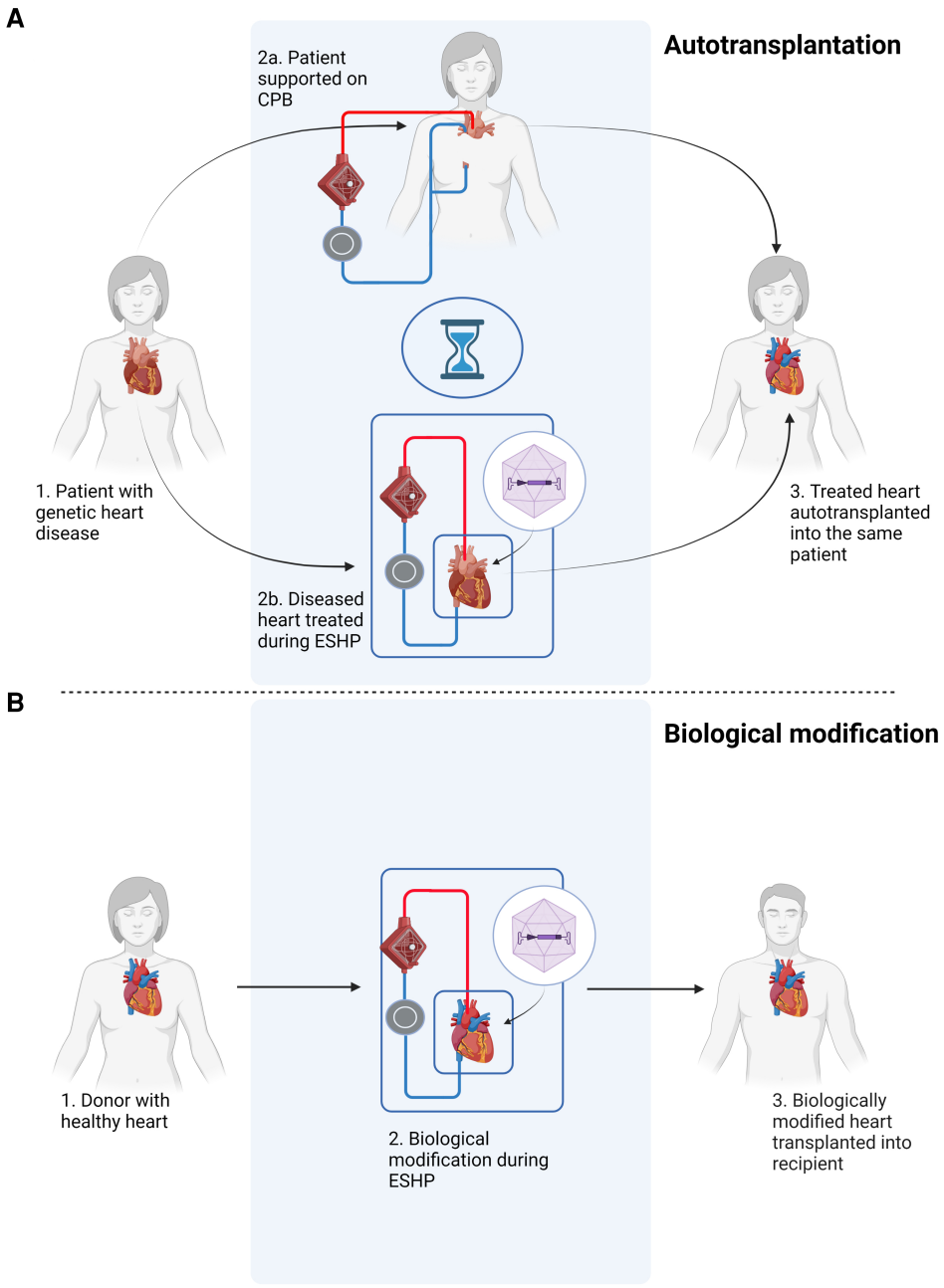

FIGURE 1
Schematic overview of potential applications of gene therapy during *ex situ* heart perfusion (ESHP). (**A**) Gene therapy of a heart with a pathogenic mutation, followed by autotransplantation. The patient is connected to a cardiopulmonary bypass (CPB) circuit during *ex situ* treatment of the diseased heart. (**B**) Gene therapy during *ex situ* heart perfusion for biological modification, e.g. immunomodulation, followed by orthotopic heart transplantation. The figure was constructed using Biorender.com.

Perfusion conditions

The identified studies universally defined hypothermic machine perfusion as perfusion of the cardiac vasculature at ±4°C. The perfusate solution consisted of acellular University of Wisconsin solution (29–31, 34, 35, 37–42), saline (36, 43) or fetal calf serum (32), and reported perfusion duration varied between 5 s ($n = 2$ studies), 15 min ($n = 6$ studies), 20 min ($n = 2$ studies), 30 min ($n = 2$ studies) and 60 min ($n = 3$ studies), with most studies using perfusion duration of 15–30 min.

When comparing continuous perfusion to a single bolus injection, multiple studies have demonstrated that transduction efficiency is superior with continuous perfusion compared to single bolus injection (34, 35, 37, 40). Evidence suggests that administration of the vector with a pulsatile perfusion pressure might further improve efficiency and transmyocardial

TABLE 1 Studies that utilize hypothermic *ex situ* heart perfusion.

Author	Year	Animal model	Used Vector	Used Gene	Perfusion setting	Results
Adenoviral vectors						
Shiraishi et al. (29)	1996	Rat	Adenovirus (AdCMVLacZ)	LacZ	20 ml of virus-containing UW solution at 4°C for 20 min at a flow of 1 ml/min with different titers and total preservation time, followed by heterotopic transplantation.	• Infection and gene delivery had no adverse effect on graft survival. • Gene expression returned to baseline after 14 days.
Gojo al. (30)	1998	Rat	Adenovirus (Adex1CALacZ)	LacZ	50 ml of virus-containing UW solution at 4°C for 60 min with different titers and total preservation time, followed by heterotopic transplantation.	• Infection and gene delivery had no adverse effect on graft survival. • Gene expression universally returned to baseline after 5 weeks. • Increased leukocyte infiltration with titers >1 × 10 (10) PFU/ml, with earlier reduction in gene expression
Abunasra et al. (31)	2003	Rat	Adenovirus (AdCMVLacZ, AdeNOS & AdMnSOD)	LacZ, eNOS, Mn-SOD	5 ml of virus-containing UW solution at 4°C for 15 min at a flow of 0.75 ml/min with different titers, followed by heterotopic transplantation.	• Infection and gene delivery had no adverse effect on contractile function. • Improved recovery of contractile functions after induced ischemia-reperfusion insult in the eNOS and Mn-SOD groups.
Yap et al. (32)	2001	Rat	Adenovirus (AdCMVLacZ)	LacZ	Virus-containing 2% fetal calf serum at 4°C for 5 s with different titers into the aortic root, followed by 60 min of storage and heterotopic transplantation.	• Increased infection and gene delivery with longer exposure times • Increased infection and gene delivery with higher titers [up to 1. × 10 (10) PFU/ml]. • Preferred gene expression in cardiomyocytes over other cell types. • Accentuated expression in areas of ischemia. • No inflammatory cell infiltration after 4 days.
Pellegrini et al. (33)	1998	Rat	Adenovirus (AdCMVLacZ)	LacZ	Virus-containing [1 × 10 (9) PFU/ml] 2% fetal calf serum at 4°C for 5 s into the aortic root, followed by 60 min of storage and heterotopic transplantation.	• Preferred gene expression in cardiomyocytes over other cell types. • Accentuated expression in areas of ischemia. • No inflammatory cell infiltration after 1 week.
Brauner et al. (34)	1997	Rabbit	Adenovirus (AdSvIL10 & AdCMVTGF-β1)	IL10, TGF-beta 1	20 ml of virus-containing UW solution at 4°C for 15 min at a flow of 1 and 0.5 ml/min, followed by heterotopic transplantation.	• Increased infection and gene delivery with continuous perfusion compared to bolus injection. • Significant IL10 and TGF-beta 1 expression in infected hearts, that increased with higher titers.
Brauner et al. (35)	1997	Rabbit	Adenovirus (AdSvIL10 & AdCMVTGF-β1)	IL10, TGF-beta 1	20 ml of virus-containing UW solution at 4°C for 15 min at a flow of 1 and 0.5 ml/min, followed by heterotopic transplantation.	• Myocardial distribution improved after increasing perfusion pressure and adding pulsatility. • Acute allograft rejection was decreased after cytokine gene therapy.
Yang et al. (36)	1999	Rat	Adenovirus (AdCTLA4Ig & AdLacZ)	LacZ, CTLA4Ig	1 ml of virus-containing [1 × 10 (11) PFU/ml] saline solution at 4°C for 10–15 min followed by heterotopic transplantation in in non-immunosuppressed mismatched rats.	• AdCTLA4Ig-treated hearts demonstrated indefinite survival in non-immunosuppressed mismatched recipients. • No lymphocytic cell infiltration was noted in the AdCTLA4Ig-treated hearts. • Gene expression was abundant in the endo-myocardium • Gene expression was not detected after 100 days.
Pellegrini et al. (37)	2000	Rat	Adenovirus (AdCMVLacZ)	LacZ	5 ml of virus-containing UW solution at 4°C for varying infection intervals with different flows and pressures, followed by heterotopic transplantation.	• Increased infection and gene delivery with continuous perfusion compared to bolus injection.

(*Continued*)

TABLE 1 Continued

Author	Year	Animal model	Used Vector	Used Gene	Perfusion setting	Results
						• Preferred gene expression in cardiomyocytes over other cell types. • Higher titers were associated with higher levels of inflammatory infiltration. • Perfusion pressures >50 mmHg were associated with more tissue damage.
Oi et al. (38)	2006	Porcine	Adenovirus (AdCMVLacZ)	LacZ	200 ml of virus containing UW solution at 4°C with varying titers, for 30 min with a perfusion pressure of 50 mmHg, followed by heterotopic heart transplantation.	• Increased infection and gene delivery with higher titers [up to 1 × 10 (9) PFU/ml]. • Homogeneous distribution across the myocardium was achieved. • Virus titers did not correlate with edema formation.
Rao et al. (39)	2007	Rat	Adenovirus (AdCMVLacZ)	LacZ	5 ml of virus-containing [3.5 × 10 (8) PFU] UW solution at 4°C for 15 min at a flow of 0.75 ml/min with different titers, followed by heterotopic transplantation.	• Infection and gene delivery had no adverse effect cardiac allograft vasculopathy development.
Abunasra et al. (40)	2006	Rat	Adenovirus (AdCMVLacZ & AdMnSOD)	LacZ, Mn-SOD	5 ml of virus-containing UW solution at 4°C for 15 min at a flow of 0.75 ml/min with different titers, followed by heterotopic transplantation.	• Increased infection and gene delivery with continuous perfusion compared to bolus injection. • Infection and gene delivery had no adverse effect on contractile function. • Improved recovery of contractile functions after induced ischemia-reperfusion insult in the Mn-SOD group.
Ricci et al. (41)	2010	Rat	Adenovirus (AdCMVhNIS)	NIS	5 ml of virus-containing [1 × 10 (9) PFU/ml] UW solution at 4°C, followed by heterotopic transplantation and injection of ^{131}I after 3 days.	• Successful gene transfer of the NIS-gene could be confirmed. • Graft survival was significantly higher in AdCMVhNIS-treated hearts following injection of ^{131}I. • No inflammatory infiltrates were found in AdCMVhNIS-treated hearts following injection of ^{131}I.
Adeno-associated viral vectors						
Miyagi et al. (42)	2008	Rat	Adeno-associated virus (rAAV9CMVLacZ)	LacZ	5 ml of virus-containing UW solution at 4°C with different titers for 20 min at a flow of 0.75 ml/min, followed by heterotopic transplantation and injection of ^{131}I after 3 days.	• Increased infection and gene delivery with higher titers (up to 2 × 10 (12 vector genomes/ml). • There was no difference in gene expression between ESHP perfusion and intravenous injection. • Durable and stable gene transfer was achieved for 3 months.
Liposome-based vectors						
Furukawa et al. (43)	2005	Rabbit	Liposome (pSVhIL-4, pSVhIL-10	IL4, IL10	10 ml of liposome-containing saline solution at 4°C for 30 min with a flow of 20 ml/min, followed by heterotopic transplantation in mismatched recipients	• Successful gene transfer could be confirmed. • Expression reached a peak at 7–8 days, followed by a slow decline. • Increased infection and gene delivery with higher titers. • No systemic wash-out was noted in recipients. • Preferred gene expression in cardiomyocytes over other cell types. • Mean allograft survival was significantly prolonged from 9 to 135 days. • There was a synergistic effect on allograft survival when both genes were delivered, potentially due to suppression of T lymphocyte infiltration induced by localized overexpression of Il4 and IL10.

(*Continued*)

TABLE 1 Continued

Author	Year	Animal model	Used Vector	Used Gene	Perfusion setting	Results
Jayakumar et al. (44)	2000	Rat	Liposome (HVJ-liposome containing HSP70 DNA)	HSP70	1 ml of liposome-containing fluid through the aortic root, followed by heterotopic transplantation and subsequent excision after 4 days for ischemia-reperfusion challenge during Langendorff perfusion.	• Successful gene transfer could be confirmed. • Improved recovery of contractile functions after induced ischemia-reperfusion insult in the HSP70 group. • Significantly higher recovery of endothelial function after induced ischemia-reperfusion insult in the HSP70 group.

UW, University of Wisconsin; PFU, plaque-forming units; eNOS, endothelial nitric oxide synthase; Mn-SOD, manganese superoxide dismutase; NIS: sodium-iodide symporter; HVJ, Hemagglutinating Virus of Japan; HSP, heat-shock protein.

distribution of the infused vector (35). Furthermore, perfusion pressure seems to be inversely associated with the required perfusion duration for satisfactory transduction, i.e., an increase in perfusion pressure results in a reduction in the time needed to achieve the same level of transduction (35, 37). It must be noted, however, that higher perfusion pressures (70–80 mmHg) are associated with increased histological tissue damage and edema formation compared to lower perfusion pressures (<50 mmHg) during hypothermic ESHP, and might damage the graft (37). Regarding edema formation, one study mentioned that Ad vector titers up to $1 \times 10(9)$ plaque-forming units (PFU)/ml did not correlate with increased edema formation. Suggesting that any edema formation observed at titers lower than $1 \times 10(9)$ PFU is most likely the result of the perfusion itself (38).

Based on the available literature, we can assume that there is a dose-dependent effect for Ad mediated gene therapy, with improved transduction at higher viral titers. However, due to heterogeneity among studies regarding specific titer definition (i.e., PFU as single vector dose, per unit perfusate volume or unit of heart weight) it is difficult to define the optimal titer range on the basis of prior literature. Furthermore, optimal titer range might also be dependent on the specific subtype of Ad (32, 34, 38). However, too high doses might result in microcirculatory obstruction [reported single vector dose $\geq 1 \times 10(11)$] (29) and an increased inflammatory response in the myocardium [reported single vector dose $\geq 1 \times 10(10)$] (30), resulting in poor myocardial function and a reduced length of gene expression (≥4 weeks with lower doses vs. 3 weeks with higher doses). The inflammatory response associated with too high doses consisted of increased leukocyte infiltration surrounding transducted cells distributed across the myocardium up to 21 days after heterotopic transplantation.(30) These findings also suggest that the level of inflammation can negatively impact duration of expression, and thus effectivity, of gene therapy during ESHP. A positive dose-dependent relationship has also been reported in liposome mediated delivery of genes (43) and with the use of AAV-based vectors (42).

In summary, these results suggest that during hypothermic ESHP continuous, pulsatile perfusion pressures with a mean pressure of 50 mmHg seem optimal. The most appropriate titer for optimal transduction is up to debate, but seems to follow a parabolic trend based on evidence that suggests a dose-dependent effect below a certain threshold, followed by a range of optimal transduction and eventually a point where higher titers are associated with graft dysfunction and reduced efficacy of transduction and expression.

Myocardial distribution

Reported myocardial distribution of transgene expression is heterogeneous and studies mainly report perfusion pressure and pulsatility as factors of influence. The effects of perfusion solution, reperfusion strategy, position of the heart and antegrade vs. retrograde perfusion on myocardial distribution was not reported. Among studies using Ad vectors, both Gojo (30), Pellegrini (33, 37) and Yap (32) reported preferred expression in cardiomyocytes over endothelial cells, while Brauner et al. (34) reported high expression in subepicardial perivascular regions (100% transduction rate) and lower in mid-wall and subendocardial regions (5%–20%). This difference in expression pattern among regions, however, was reduced by increasing perfusion pressure and adding pulsatility, resulting in a more equal distribution (25%–40%) across the myocardium. On the contrary, Yang et al. (36) report abundant expression in the endomyocardial tissue, but lower in the mid layers of myocardium using an antegrade perfusion approach. They did not report on specific perfusion pressure to explain these differences in distribution. Pellegrini et al. (33, 37) also report accentuated transgene expression in the right ventricle, especially in the subepicardial region, upon infusion of $1 \times 10(9)$ PFU/ml Ad vector at different perfusion pressures (up to 40–50 mmHg) and exposure times. They also noted increased expression around zones that endured (warm) ischemia, possibly due to locally increased endothelial permeability associated with (warm) ischemia (33). Oi et al. (38) report a homogeneous distribution of expression across a multitude of left ventricular segments after using a continuous perfusion pressure of 50 mmHg, suggesting that heterogeneous distribution is less of a concern when higher perfusion pressures are used. In models using liposome-mediated transfer, one study reported homogeneously distributed transfection after 30 min of cold perfusion at a non-specified perfusion pressure

TABLE 2 Studies that utilize normothermic *ex situ* heart perfusion.

Author	Year	Animal model	Used Vector	Used Gene	Perfusion setting	Results
Adenoviral vector						
Donahue et al. (45)	1997	Rabbit	Adenovirus (AdCMVLacZ)	LacZ	50 ml of virus-containing oxygenated KHB at 37°C, flows between 10 and 40 ml/min, pressures between 10 and 70 mmHg, varying infection intervals up to 180 min.	• Increased infection and gene delivery at flows >30 ml/min. • Increased infection and gene delivery with longer exposure times. • Increased infection and gene delivery with higher virus titers [up to 1.6 × 10 (9) PFU/ml].
Donahue et al. (46)	1998	Rabbit	Adenovirus (AdCMVLacZ)	LacZ	Virus-containing oxygenated KHB at 37°C with varying flows, pressures and infection intervals, for a total Langendorff time of 180 min. Pretreatment with calcium-KHB, KHB supplemented with bradykinin, serotonin, L-NAME, heparinized blood.	• Increased infection and gene delivery with longer exposure times. • Increased infection and gene delivery with higher titers [up to 1.6 × 10 (9) PFU/ml]. • Increased infection and gene delivery after pretreatment with agents that increase microvascular permeability. • Synergistic effect after combination of the above mentioned factors.
Nagata et al. (47)	2001	Rabbit	Adenovirus (AdCMVLacZ)	LacZ	Virus-containing [1 × 10 (8)PFU/ml oxygenated KHB at 37°C. Infection interval was fixed at 2 min, for a total Langendorff time of 180 min. Pretreatment with VEGF, TNG, 8Br-cGMP, L-NMMA, ODQ, sildenafil, zaprinast.	• Increased infection and gene delivery after pretreatment with agents that increase available nitric oxide. • Synergistic effect after combination of agents that increase available nitric oxide.
Lehnart et al. (48)	2000	Rabbit	Adenovirus (AdCMVLacZ & AdRSVLuc)	LacZ & Luciferase	25 ml of virus-containing [1.6 × 10 (9) PFU/ml] oxygenated KHB at 37°C recirculated for 60 min at a flow of 30 ml/min.	• No adverse effect on contractile and diastolic properties over 48 h of functional evaluation. • Infection and gene delivery had no adverse effect on response to beta-adrenergic stimulation.
Bishawi et al. (49)	2019	Porcine	Adenovirus (AdCMVLuc)	Luciferase	Virus-containing [5 × 10 (13) PFU/ml] solution mixed with crystalloid prime and washed RBC's, perfusion for 2 h at a flow of 600 ml/min, perfusion pressure of 65–70 mmHg, followed by flushing and heterotopic transplantation.	• Complete inhibition of infection and gene delivery by plasma and serum. This effect was minimized by using washed RBC's in combination with crystalloid prime. • Successful infection and gene delivery across all areas of the heart, as well as the coronary arteries. • Accentuated infection and gene delivery in right ventricular and septal areas when compared to the left ventricle. • No systemic wash-out after flushing and heterotopic transplantation
Adeno-associated viral vectors						
Mendiola Pla et al. (50)	2023	Porcine	Adeno-associated virus (AAV3b SASTG)	Luciferase	Virus-containing solution with different titers mixed with crystalloid prime and washed RBC's, perfusion for 2 h at a flow of 600 ml/min, perfusion pressure of 60–70 mmHg, followed by flushing and heterotopic transplantation.	• High transduction efficiency of SASTG • Most uptake (83.82%) within the first 30 min • Solid transgene expression up to 35 days without off-target effects or signs of rejection • Dose-dependent effect.

KHB, krebs-henseleit buffer; PFU, plaque-forming units; L-NAME, N(ω)-nitro-L-arginine methyl ester; VEGF, vascular endothelial growth factor; TNG, trinitroglycerin; 8Br-cGMP, 8-Bromoguanosine 3′,5′-cyclic monophosphate; L-NMMA, N^G-monomethyl-L-arginine; ODQ, Oxadiazolo-[4,3-a]-quinoxalin-1-one.

(43). No reports on myocardial distribution were identified using AAV.

Taken together, these results suggest that pulsatile perfusion pressures centered around a mean pressure of 50 mmHg seems optimal to achieve a homogeneous distribution during hypothermic ESHP.

Therapeutic interventions

Regarding therapeutic potential, some studies assessed the efficacy of gene therapy during ESHP for immunomodulation, or to increase tolerance of the graft to ischemia-reperfusion injury (**Table 3**; **Figure 2**). One study assessed the influence of Ad vectors on the development of cardiac allograft vasculopathy.

TABLE 3 Studies that investigated the therapeutic potential of gene therapy during hypothermic ESHP.

Author	Year	Animal model	Used Vector	Used Gene	Perfusion setting	Results
Adenoviral vectors						
Brauner et al. (34)	1997	Rabbit	Adenovirus (AdSvIL10 & AdCMVTGF-β1)	IL10, TGF-beta 1	20 ml of virus-containing UW solution at 4°C for 15 min at a flow of 1 and 0.5 ml/min, followed by heterotopic transplantation.	• Significant IL10 and TGF-beta 1 expression in infected hearts, that increased with higher titers.
Brauner et al. (35)	1997	Rabbit	Adenovirus (AdSvIL10 & AdCMVTGF-β1)	IL10, TGF-beta 1	20 ml of virus-containing UW solution at 4°C for 15 min at a flow of 1 and 0.5 ml/min, followed by heterotopic transplantation.	• Acute allograft rejection was decreased after cytokine gene therapy.
Yang et al. (36)	1999	Rat	Adenovirus (AdCTLA4Ig & AdLacZ)	LacZ, CTLA4Ig	1 ml of virus-containing [1 × 10 (11) PFU/ml] saline solution at 4°C for 10–15 min followed by heterotopic transplantation in in non-immunosuppressed mismatched rats.	• AdCTLA4Ig-treated hearts demonstrated indefinite survival in non-immunosuppressed mismatched recipients. • No lymphocytic cell infiltration was noted in the AdCTLA4Ig-treated hearts.
Abunasra et al. (31)	2003	Rat	Adenovirus (AdCMVLacZ, AdeNOS & AdMnSOD)	LacZ, eNOS, Mn-SOD	5 ml of virus-containing UW solution at 4°C for 15 min at a flow of 0.75 ml/min with different titers, followed by heterotopic transplantation.	• Improved recovery of contractile functions after induced ischemia-reperfusion insult in the eNOS and Mn-SOD groups.
Abunasra et al. (40)	2006	Rat	Adenovirus (AdCMVLacZ & AdMnSOD)	LacZ, Mn-SOD	5 ml of virus-containing UW solution at 4°C for 15 min at a flow of 0.75 ml/min with different titers, followed by heterotopic transplantation.	• Infection and gene delivery had no adverse effect on contractile function. • Improved recovery of contractile functions after induced ischemia-reperfusion insult in the Mn-SOD group.
Ricci et al. (41)	2010	Rat	Adenovirus (AdCMVhNIS)	NIS	5 ml of virus-containing [1 × 10 (9) PFU/ml] UW solution at 4°C, followed by heterotopic transplantation and injection of ^{131}I after 3 days.	• Graft survival was significantly higher in AdCMVhNIS-treated hearts following injection of ^{131}I. • No inflammatory infiltrates were found in AdCMVhNIS-treated hearts following injection of ^{131}I.
Liposome-based vectors						
Jayakumar et al. (44)	2000	Rat	Liposome (HVJ-liposome containing HSP70 DNA)	HSP70	1 ml of liposome-containing fluid through the aortic root, followed by heterotopic transplantation and subsequent excision after 4 days for ischemia-reperfusion challenge during Langendorff perfusion.	• Improved recovery of contractile functions after induced ischemia-reperfusion insult in the HSP70 group. • Significantly higher recovery of endothelial function after induced ischemia-reperfusion insult in the HSP70 group.
Furukawa et al. (43)	2005	Rabbit	Liposome (pSVhIL-4, pSVhIL-10	IL4, IL10	10 ml of liposome-containing saline solution at 4°C for 30 min with a flow of 20 ml/min, followed by heterotopic transplantation in mismatched recipients	• Mean allograft survival was significantly prolonged from 9 to 135 days. • There was a synergistic effect on allograft survival when both genes were delivered, potentially due to suppression of T lymphocyte infiltration induced by localized overexpression of IL4 and IL10.

UW, University of Wisconsin; PFU, plaque-forming units; eNOS, endothelial nitric oxide synthase; Mn-SOD, manganese superoxide dismutase; NIS, sodium-iodide symporter; HVJ, Hemagglutinating Virus of Japan; HSP, heat-shock protein.

A total of 5 studies have shown that it is possible to modulate the immune response associated with allograft implantation. Brauner et al. (34) constructed Ad vectors expressing viral interleukin-10 (AdSvIL10) or transforming growth factor-beta 1 (AdCMVTGF-beta 1), two anti-inflammatory cytokines, and delivered them to rabbit hearts. Following ESHP, the hearts were heterotopically transplanted in recipient rabbits. They reported successful expression of TGF-beta 1 and IL10 in the grafts, especially with higher vector concentrations, and no evidence of neoplastic side-effects, such as intimal proliferation, or pro-fibrotic effects, assessed after 4 days of follow-up. In a subsequent study (35), using the same model, the authors showed a significant inhibitory action on acute allograft rejection through immunosuppressive cytokine gene delivery, prolonging graft survival. Building on this experience, Yang et al. (36) used Ad vectors (AdCTLA4Ig) to deliver the CTLA4Ig gene to promote survival of grafts in a heterotopic transplantation model of Lewis and Brown Norway rat hearts. They reported abundant cardiac expression of the CTLA4Ig transgene after transplantation in non-immunosuppressed Wistar Furth recipients. Although progressive diminution of CTLA4Ig mRNA expression was noted over time, the allografts survived indefinitely with a sufficient degree of

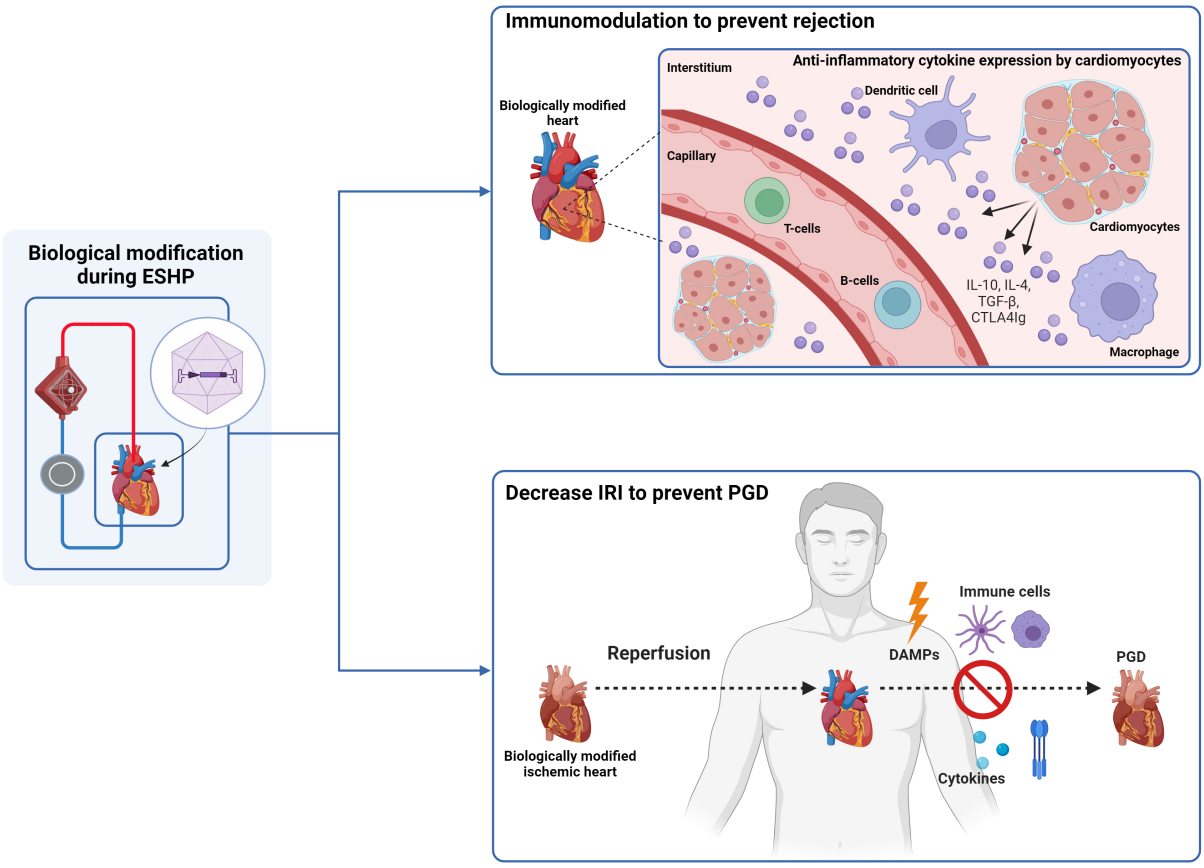

FIGURE 2
Gene therapy during ESHP has been investigated for immunomodulation and to decrease ischemia-reperfusion injury (IRI). Immunomodulation was conducted by transduction of cardiomyocytes with adenoviral vectors that carried genes encoding anti-inflammatory cytokines (IL-4, IL-10, TGF-beta, and CTLA4IG. IRI was attenuated by adenoviral mediated gene transfer of manganese superoxide dismutase, nitric oxide synthase and heat shock protein 70. ESHP: ex situ heart perfusion. IL: interleukine. PGD: primary graft dysfunction. DAMPS: damage-associated molecular pattern. The figure was constructed using Biorender.com.

localized immunosuppression. Ricci et al. (41) studied the effects of gene transfer of human iodide symporter and subsequent treatment with ^{131}I on acute allograft rejection using Ad vectors in a heterotopic rat transplantation model. They demonstrated significantly longer survival and reduced myocardial damage in grafts perfused with the Ad vectors containing the gene that received treatment with ^{131}I. Finally, Furukawa et al. (43) used a liposomal-mediated approach to deliver a combination of IL4 and IL10 in a heterotopic transplantation model of rabbit hearts. They reported successful gene transfer and were able to prolong allograft survival in hearts that received both interleukins (9 ± 2 days vs. 135 ± 3 days) with a great improvement of histological rejection grades, indicating a synergistic action of both cytokines. This was presumably due to reduced alloreactivity of T-lymphocytes induced by localized overexpression of the interleukins. By reducing this alloreactivity, long-term survival of cardiac allografts without systemic immunosuppression was possible. Taken together, these results indicate that (local) immunomodulation of transplanted grafts is possible during ESHP, which might enable a less restrictive immunosuppressive regimen to prevent rejection in recipients.

A total of three studies demonstrated that the authors were able to enhance myocardial tolerance to ischemia-reperfusion injury, preserving both contractile and endothelial function of treated grafts. Abunasra et al. (31, 40) used a heterotopic transplantation model to demonstrate the protective effects of Ad mediated gene transfer of manganese superoxide dismutase and nitric oxide synthase into rat hearts. After heterotopic transplantation, the hearts were procured and reperfused on a Langendorff system for assessment and induction of an ischemic insult. After demonstrating successful gene transfer and expression, they noted improved recovery of contractile function after the ischemic insult in treated hearts. Jayakumar et al. (44) used a similar model to assess the effects of heat shock protein 70 (Hsp70) gene transfection on rat hearts using liposomal vectors for delivery. They demonstrated improved postischemic recovery of contractile function and recovery of coronary flow, together with reduced creatinine kinase release in hearts that were treated with Hsp70. These results indicate that myocardial ischemia-reperfusion injury can potentially be attenuated using gene therapy.

Finally, Rao et al. (39) used a heterotopic heart transplantation model to study the effects of Ad mediated gene transfer on the later

development of cardiac allograft vasculopathy due to concerns over the potential effects of adenoviral therapy on the development of allograft vasculopathy. They concluded that Ad mediated gene transfer did not result in accelerated allograft vasculopathy development compared to non-treated controls after 120 days.

Gene therapy during normothermic *ex situ* heart perfusion

Gene therapy research during normothermic ESHP mostly involved Ad mediated gene transfer (45–49) using rabbit (45–48) or porcine hearts (49). One study involved AAV mediated gene transfer in porcine hearts (50). The identified studies were notably lower in number compared to studies that assessed gene therapy during hypothermic ESHP (6 vs. 17).

Donahue et al. (45, 46) studied the optimal conditions for delivery of gene products during normothermic ESHP using Ad vectors in rabbit hearts. They found that transduction increased incrementally with coronary flow (up to 40 ml/min), exposure time (up to 120 min) and administered viral dose [up to 1.6×10 (9) PFU/ml] (45). They also report further improvements in efficacy by minimizing calcium concentration in the perfusate and implementing pretreatment of the hearts with several pharmacological agents that increase microvascular permeability, such as bradykinin, serotonin and L-NAME. As an example they noted a transduction rate of over 90% in just two minutes of perfusion by combining hypocalcemia and serotonin administration, confirming the hypothesis that required exposure time could dramatically be reduced by modulating viral dose and vascular permeability (46). In a follow-up study using the same model, Nagata et al. (47) report that pretreatment with vascular endothelial growth factor combined with a phosphodiesterase inhibitor, such as nitroglycerin or sildenafil, could also increase the efficiency of gene transfer in a dose-dependent fashion. Furthermore, Lehnart et al. (48) demonstrated that transduction of cardiomyocytes with Ad vectors does not adversely affect contractile function of perfused hearts, strengthening the belief that gene therapy using Ad vectors has potential as a safe modality for therapeutic intervention. Further proof-of-concept was provided by Bishawi et al. (49), who demonstrated safe and efficacious Ad mediated gene transfer in a porcine heterotopic transplantation model. In this study, the authors used 2 h of normothermic ESHP as a platform to deliver $5 \times 10(13)$ total viral particles of an Ad luciferase vector (AdCMVLuc) prior to allograft implantation in a blood type compatible recipient pig. Enzymatic assessment of luciferase activity obtained 5 days after transplantation revealed global and uniform luciferase activity in the allograft and coronary arteries, without systemic off-target expression. Interesting lessons learned during these experiments include the apparent inhibitory influences of plasma and serum on transduction efficiency. However, these inhibitory effects could be minimized when cell-salvaged erythrocytes were mixed with the priming solution instead of whole blood, although some inhibitory effects on transduction efficiency remained. Nevertheless, by using a perfusate solution based on crystalloid prime and cell-salvaged erythrocytes, they reported satisfactory expression across all areas of the heart after heterotopic transplantation into recipient pigs. Based on these findings, the authors conducted a subsequent study into the efficacy of AAV based delivery and transduction during ESHP using a similar model (50). Their most important finding was that they were able to achieve durable transgene expression using AAV-mediated gene transfer for up to 35 days following heterotopic transplantation, without signs of systemic off-target expression, rejection or inflammation in the graft. Furthermore, they identified SASTG, a myocardial-enhanced AAV3b variant, as the most efficient vector to deliver transgenes when used during normothermic ESHP. Regarding kinetic profile, they reported that most transduction occurred within the first 30 min of perfusion and confirmed the existence of a dose-dependent response, with increased transduction rates with incremental titers infused.

Discussion

The results summarized in this review highlight the feasibility and clinical potential of cardiac gene therapy during ESHP, especially considering the fact that both hypothermic (51) and normothermic (7, 8) ESHP have already been introduced in clinical practice. The evaluation of gene therapy in both temperature ranges is an interesting challenge by itself. Contrary to what might be expected, successful transduction could be achieved under hypothermic conditions, followed by expression of the inserted gene after heterotopic transplantation. This may be surprising, since hypothermia is associated with a significant reduction of enzymatic activity, reduced cellular respiration and metabolism (52), and, subsequently, reduced genetic processing of nucleic-acids used for genomic modification. Although the latter might be the result of subsequent rewarming (and hence restoration of metabolism with subsequent processing of the delivered genetic material) after transplantation, these result indicate that hypothermia does not have to limit vector viability or entry into cardiomyocytes and suggest that normothermic metabolism isn't a prerequisite for successful transduction. This is supported by the prolonged expression that was noted after gene therapy during hypothermic ESHP in multiples studies (36, 41–43). Furthermore, effective transduction during hypothermic ESHP might also be the result of the absence of components in the perfusate solution that negatively influence vector delivery, such as and plasma, since all studies use a crystalloid-based perfusate. Nonetheless, these results can be interpreted as evidence that the association between metabolism and efficacy of gene therapy might be more complex in nature than expected and also dependent on multiple other factors, like the specific vector used (53, 54) and composition of the perfusate solution (cellular vs. acellular).

Another important finding is that perfusion conditions, such as perfusion pressure and viral dose, seem to impact the efficiency of transduction in both hypothermic and normothermic conditions. Although heterogeneity regarding optimal vector dose is quite

substantial, a pulsatile perfusion pressure around a mean of 50 mmHg seems required for optimal vector delivery and transduction. Endothelial integrity also plays an important role. Under normal physiological circumstances, the endothelium constitutes an uninterrupted barrier between the intravascular and extravascular compartment. When the endothelium becomes activated by pathological triggers, such as inflammation or excessively high pressures, gaps start to occur between the cells that facilitate diapedesis of leukocytes and contributes to edema formation. In the context of gene therapy, however, a certain degree of endothelial activation seems to be a prerequisite for success, as is evidenced by the finding that transduction efficiency during normothermic perfusion is improved by the addition of agents that increase microvascular permeability, a phenomenon that has also been previously observed for *in vivo* delivery studies (55). The harvesting of a donor heart is inherently associated with some degree of (warm) ischemia, even when ESHP is utilized. The subsequent reperfusion-associated injury will result in a varying degree of endothelial permeability (56, 57), which facilitates efficient gene transfer. This might hypothetically obviate the need for supplementation of agents that affect microvascular permeability in clinical situations that are associated with substantial (warm) ischemia, although this should be investigated in future studies. Increased microvascular permeability might also partially explain the observed transduction rates during hypothermia, as hypothermia in itself is associated with reversible morphological and functional changes to the endothelium that increase permeability (58). These findings are in accordance with what is known from studies that assess vector uptake in the myocardium during *in vivo* administration (17). However, given the fact that the above is investigated in preclinical models, one should be aware of a potential translational gap between small and large animal models and clinical efficacy in humans, as is evidenced by multiple clinical studies that failed to demonstrate efficacy in humans after convincing preclinical results in small and large animals (17, 20, 22). Future research, specifically designed to address these questions, seems a prerequisite before successful clinical implementation.

Central to the successful application of gene therapy, is efficiency of delivery (20). Efficiency of delivery in itself is dependent on the vector and the route of administration, both of which can be extensively controlled and manipulated during ESHP. Currently, potential vectors can be subdivided into viral and non-viral vectors. Non-viral vectors include liposomes, while extensive experimentation has indicated that two viral vector systems are effective at cardiac gene transfer, being Ad vectors and AAV vectors (13, 20). Generally speaking, Ad vectors have a high efficiency for delivery and expression of their genome within the target cells. They are effectively produced at high titers, are associated with rapid gene expression kinetics and can carry large genes due to their substantial insert capacity, in particular when considering the use of third generation, so-called "gutless" Ad vectors (59). However, important disadvantages are the significant associated immune response, triggering both innate and adaptive immunity which limits the duration of gene expression, and their innate tropism for many human tissues other than the heart, which can be circumvented during ESHP (17, 20). On the other hand, AAV vectors have the advantage that they are principally non-pathogenic and can have a high tropism for myocardium depending on serotype (60–62). They are also associated with sustained transgene expression. Disadvantages include their limited insert capacity, limiting the size of genes that can be transported, and relatively high production costs. They are also quite prone to evoking an immune response, antibody development and it seems that a large proportion of the general population seems to possess neutralizing antibodies for different AAV serotypes before treatment (63, 64), although this might be less of a problem during an ESHP approach. Based on the above, one can imagine that selection of the appropriate vector for delivery of the desired gene is not a straightforward process and depends upon many factors that influence outcome, including intention of treatment. Based on the available literature, Ad vectors currently seem the preferred vector for gene delivery during ESHP due to their large gene carrying capabilities, efficiency of transduction, the relatively low costs and favorable expression kinetics, which abrogates the theoretical advantages of AAV over Ad in the setting of ESHP. The higher immunogenic response that is associated with Ad therapy might also not be an issue during ESHP as it is possible to use a leukocyte-depleted perfusate, which may be supplemented with additional immunosuppressants (65), or use a perfusion approach that might not need any blood constituents, such as hypothermic ESHP. However, the limited long-term expression rate of genes delivered with Ad vectors might be an important barrier to long-lasting effects of gene therapy to treat pathogenic genetic mutations in carriers, unless they are used for gene-editing purposes (e.g., CRISPR-CAS). For gene delivery, however, this might be an important argument for the use of AAV over Ad in situations where a long-lasting expression of gene products is warranted, e.g., for replacing certain genetic defects or when long-lasting immunosuppression is desired (66), especially with the development of highly cardioselective serotypes, such as SASTG, that could greatly enhance efficiency of transduction (17, 20, 22, 67).

If implemented correctly, ESHP offers a unique opportunity for direct biological modification of a (defective) heart without the risks of systemic toxicity or side-effects. By isolating the organ in a metabolically and immunologically favorable condition, it is possible to directly investigate and manipulate the factors that influence important obstacles related to delivery and uptake that were encountered during *in vivo* clinical trials and potentially improve the success rate of cardiac gene therapy. By doing so, ESHP opens up the possibilities to improve quality of grafts, allow for selective immunomodulation to minimize the need for immunosuppression, improve tolerance to ischemia-reperfusion injury and further extend the donor pool. Ultimately, it could be used for correction of a genetic defect in known carriers of a pathogenic mutation after careful excision of the heart, preservation by machine perfusion and autotransplanting it into the same patient, which is supported by cardiopulmonary bypass during ESHP. By identifying and selectively treating these

patients at an early stage, this could potentially ameliorate the need for heart transplantation altogether, thereby improving the current supply-demand mismatch and catalyzing a transition towards a new era of regenerative medicine and organ transplantation. Furthermore, lessons learned from experience with gene therapy during ESHP could also be employed in newly developed techniques for loco-regional isolated *in situ* heart perfusion, as previously described by White et al. (28) and which are currently being developed for clinical application (e.g., DiNAQOR AG (Schlieren, Switzerland). Especially in the light of recent developments in gene-editing techniques that might benefit from direct, isolated exposure to the target organ (e.g., CRISPR-CAS, TALENs) (68–70) and the recent successes in clinical application of gene therapy in other fields (71–74), these future prospects might be closer to reality than initially anticipated.

To summarize, key messages from the literature regarding the application of gene therapy during ESHP are that gene therapy is possible in both hypothermic and normothermic conditions, using Ad, AAV and liposomes. Perfusion conditions, such as pressure, duration of exposure to the vector, dose and perfusion composition seem to influence efficiency of transduction, while some degree of microvascular permeability is a prerequisite to successful application. To date, local immunomodulation and enhanced myocardial tolerance to ischemia-reperfusion injury have been achieved using gene transfer during ESHP in rodent models. Future studies should focus on replicating these findings in large animal models and humans, and the efficacy of gene therapy for the treatment of known mutations that affect heart function, such as mutations in phospholamban, lamin A/C, PKP2 or titin genes, using ESHP.

Author contributions

MV: Writing – original draft, Writing – review & editing. JA: Writing – original draft. EB: Writing – review & editing. PD: Writing – review & editing. JS: Writing – review & editing. MM: Writing – review & editing. GB: Writing – review & editing. DB: Writing – review & editing. NK: Writing – review & editing.

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Conflict of interest

GB reports ownership interest in PacingCure BV.

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Supplementary material

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Heart graft preservation technics and limits: an update and perspectives

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Heart transplantation, the gold standard treatment for end-stage heart failure, is limited by heart graft shortage, justifying expansion of the donor pool. Currently, static cold storage (SCS) of hearts from donations after brainstem death remains the standard practice, but it is usually limited to 240 min. Prolonged cold ischemia and ischemia-reperfusion injury (IRI) have been recognized as major causes of post-transplant graft failure. Continuous ex situ perfusion is a new approach for donor organ management to expand the donor pool and/or increase the utilization rate. Continuous ex situ machine perfusion (MP) can satisfy the metabolic needs of the myocardium, minimizing irreversible ischemic cell damage and cell death. Several hypothermic or normothermic MP methods have been developed and studied, particularly in the preclinical setting, but whether MP is superior to SCS remains controversial. Other approaches seem to be interesting for extending the pool of heart graft donors, such as blocking the paths of apoptosis and necrosis, extracellular vesicle therapy, or donor heart-specific gene therapy. In this systematic review, we summarize the mechanisms involved in IRI during heart transplantation and existing targeting therapies. We also critically evaluate all available data on continuous ex situ perfusion devices for adult donor hearts, highlighting its therapeutic potential and current limitations and shortcomings.

KEYWORDS

PGF primary graft failure, MP machine perfusion, ESNP ex situ normothermic perfusion, heart tranplantation, heart preservation, hypothermic machine perfusion (HMP)

Abbreviations

ATP, adenosine triphosphate; BSD, brainstem death; DCD, donation after circulatory death; PGF, primary graft failure; MP, machine perfusion; ECMO, veno-arterial extracorporeal membrane oxygenation; ESNP, *ex situ* normothermic perfusion; HIF1, hypoxia inducible factor 1; HMP, hypothermic machine perfusion; HT, heart transplantation; IPC, ischemic preconditioning; IRI, ischemia-reperfusion injury; NO, nitric oxide; NOS, nitric oxide synthase; NRP, normothermic regional perfusion; PGI2, prostaglandin 2; ROS, reactive oxygen species; SCS, static cold storage.

Introduction

Heart transplantation (HT) serves as the gold standard therapy for advanced heart failure (1). The global count of HT procedures has consistently risen each year, surpassing 5,000 cases (2). Nevertheless, this figure remains constrained due to the scarcity of available organ donors, a scenario in which demand far outpaces supply. In France, the shortage of donors remains relatively constant, with only 1 donor for every 2–2.25 transplant candidates (3). This scarcity significantly restricts the viability of HT (4).

Consequently, grafts sourced from extended criteria donors are now under consideration. These donors are typically over the age of 55 years, possess mild left ventricular hypertrophy, exhibit non-obstructive coronary artery disease, are recipients of high doses of vasopressors/inotropes, or show indications of left ventricular dysfunction due to brain death interaction (5–7). In addition, the complexity of recipients is on the rise, with a greater prevalence of comorbidities, redo surgeries, and pre-transplant veno-arterial extracorporeal membrane oxygenation (ECMO). Furthermore, there have been recent alterations to graft allocation strategies in France and the United States of America that are associated with more frequent pretransplant ECMO usage and less favorable outcomes when considering waiting list mortality and post-transplant mortality. However, these effects may not be evident in every dataset and country (8–12). For example, among patients who underwent transplantation between 2010 and 2017, the highest risk of 1-year mortality was linked to the need for end-organ support with ECMO (HR 1.59) and mechanical ventilation (HR 2.11) (13).

The evolving scenario of marginal graft acquisition and the growing complexity of recipients have extended the duration of cold ischemia and subsequent ischemia-reperfusion injury (IRI), both of which contribute to a heightened risk of primary graft failure (PGF) (1, 3). PGF is generally defined by the requirement for high-dose inotropes and/or mechanical support immediately following transplantation. As per the International Society for Heart and Lung Transplantation registry, survival rates diminish with prolonged ischemic periods (14).

Current standard practice involves static cold storage (SCS) for hearts from donors following brain death. This method combines cardioplegia and hypothermia, which substantially reduces the energy needs of the donor heart. Nevertheless, an ischemic time (between aortic clamping in the donor until aortic declamping in the recipient) exceeding 240 min is linked to an elevated risk of PGF (OR 3.01) (15, 16).

Allograft injury can manifest as transient myocardial stunning, which lasts for 12–24 h post-HT (17), or as definitive myocardial stunning (18). However, PGF remains the primary cause of early mortality, accounting for up to 40% of deaths within the initial 30 days after transplantation. The incidence varies between centers based on the definition and acceptance criteria for grafts and ranges from 15% to 40% (3, 19–21).

Ischemia reperfusion injury in heart transplantation

Currently, the standard approach involves utilizing SCS for hearts obtained following brainstem death (BSD). However, this method is linked to significant occurrences of IRI (16, 18). Recent advancements in understanding IRI have brought this syndrome to the forefront of transplantation discussions. The mechanisms underlying IRI operate at both the organ and cellular level through hypoxia and re-oxygenation processes, which play a substantial role in various pathophysiological processes that contribute to the onset of PGF. Integrating this newfound knowledge about IRI is particularly crucial in the context of HT, which still grapples with prolonged periods of ischemia and the immediate repercussions of IRI on cardiac function upon organ reperfusion.

The cellular and molecular mechanisms underlying IRI are intricate and diverse. Among these, impaired mitochondrial function and the depletion of energy metabolites are particularly important for the heart given its close interdependence with energy metabolism. A synthesis of the principal mechanisms and some of their resultant effects is presented in **Table 1**. It is vital to acknowledge that these processes exhibit high levels of interconnectedness (as illustrated in **Figure 1**) and are influenced by other factors, such as brain death, donor age, or the pre-existing presence of cardiomyopathy.

Consequences of brain death

Brain death has a significant impact on heart graft function and transplantation. It precipitates an intense systemic inflammatory response often referred to as the "cytokine storm". This inflammatory response is characterized by an excessive activation of the immune system, leading to the release of inflammatory mediators (cytokines, chemokines, coagulation factors) into the blood.

Myocardial damage resulting from this cytokine storm can compromise the heart's ability to function properly after transplantation. This can manifest as reduced ventricular

TABLE 1 Cellular and molecular processes during IRI.

Damages	Consequences
Oxygen and energy substrate deprivation	High-energy substrates deprivation
Mitochondrial function failure	Damage of oxidative phosphorylation
	Damage of mitochondrial permeability
	Cytochrome C release
	Apoptosis
Ionic damage	Increase of Ca^+, Na^+, H^+
Endothelial damage	Microvascular damage, nitric oxide loss (NO)
Oxidative stress	Reactive oxygen species production
	Protein oxidation
	Immune system stimulation
Inflammation	Immune system stimulation, vasculopathy

Ca^+, calcium ion; Na^+, sodium ion; H^+, proton; NO, nitric oxide.

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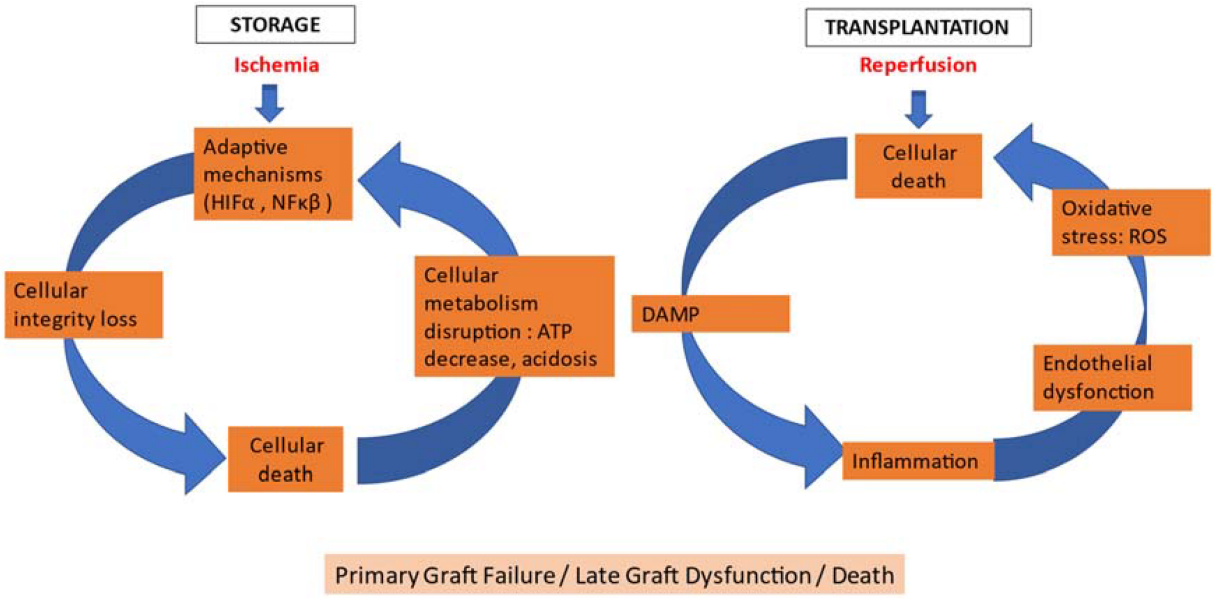

FIGURE 1
Ischemia-reperfusion process. Ischemia reperfusion: major cellular and clinical consequences in heart transplantation. DAMP: damage-associated molecular pattern; HIF1α: hypoxia inducible factor 1α; NF$\kappa\beta$: nuclear factor kβ; ROS: reactive oxygen species.

function, decreased contractility, and an increased susceptibility to IRI during transplantation.

Additionally, the period of brain death may be associated with hemodynamic and metabolic disturbances in the donor, which can also impact cardiac function.

Ischemia

Ischemia is traditionally defined as a mismatch between the supply of oxygen and energy substrates, and the cellular requirements for proper function and survival. This disparity can lead to various forms of cellular damage. In the context of heart transplantation, the ischemia phase is initiated after the donor's aortic clamp, when the heart is no longer perfused.

Mitochondrial damage

In the presence of insufficient oxygen supply, mitochondria are unable to carry out oxidative phosphorylation, a vital process for producing adenosine triphosphate (ATP). Consequently, anaerobic glycolysis becomes the primary means of generating ATP, but the levels are inadequate for fulfilling the cellular energy demands. Prolonged hypoxia results in an ATP deficit that hampers the activity of essential transporters, such as the Na^+/K^+-ATPase pump. Consequently, cytoplasmic sodium (Na^+) levels increase, with a corresponding reduction in potassium (K^+). This heightened Na^+ concentration triggers cellular swelling, loss of structural integrity, and the activation of Na^+/Ca^{2+} channels, causing an elevation in cytoplasmic calcium (Ca^{2+}) levels (22). The excessive influx of calcium due to elevated Na^+ and inefficient Ca^{2+} removal from the cytoplasm plays a pivotal role in the context of IRI. This surge in calcium activates enzyme systems reliant on calcium, contributing to the initiation of pro-inflammatory processes through the synthesis of lipid mediators, such as prostaglandins. Moreover, this calcium influx triggers the activation of proteases, which disrupt the cytoskeleton and promote cell apoptosis, ultimately leading to cell death.

Oxidative stress

Oxidative stress is characterized by an excessive production of reactive oxygen species (ROS), which is often observed during re-establishment of the oxygen supply. The generation of ROS triggers substantial cellular damage, primarily through processes such as lipid peroxidation, which in turn leads to sterile inflammation, causing vascular permeability. Furthermore, ROS production contributes to impairment of mitochondrial function. The modifications in mitochondrial structure, coupled with calcium accumulation and the generation of free radicals, initiate apoptosis via mechanisms involving increased membrane permeability, resulting in opening of the mitochondrial transition pore; the release of cytochrome C; and the subsequent activation of caspase 9, followed by other apoptotic proteases. These processes are modulated by the Bcl-2 protein family situated within the mitochondrial membrane. Nevertheless, when ischemic conditions prevail, adaptive responses to hypoxia manifest (23). An enzyme called xanthine oxidase, which is activated by elevated levels of calcium ions, is responsible for generating superoxide anions under such circumstances. The renowned mechanism relies on the activity of the heterodimeric transcription factor hypoxia-inducible factor 1 (HIF1), which becomes activated in response to decreased tissue oxygen levels. HIF1 triggers the expression of proteins involved in various

processes, including erythropoiesis, angiogenesis, and metabolic adaptation.

Endothelial damage

The primary target of ischemic conditions is the endothelial cell. The effects of ischemia on endothelial cells significantly impact the vascular damage that contributes to the long-term outcome of heart grafts. These damages arise from various factors, such as hypothermia, elevated intracellular calcium (Ca^{2+}) levels, or the composition of graft preservation solutions (e.g., containing high potassium concentrations). Under normal physiological conditions, the endothelium facilitates the release of molecules that regulate the homeostasis of the vascular wall. These molecules include nitric oxide (NO), prostaglandin (PGI2), and endothelium-derived hyperpolarizing factor. However, ischemia disrupts the endothelium's ability to regulate these functions, particularly by altering the expression and activity of nitric oxide synthases (NOSs), leading to a reduction in NO production (24).

Endothelial cells lose their ability to maintain the integrity of the endothelial barrier under ischemic conditions. However, they become activated, resulting in the expression of cytokines, chemokines, receptors, and adhesion molecules that are responsible for recruiting leukocytes. These processes play a crucial role in the formation of lesions during the reperfusion phase (25). Ultimately, prolonged ischemia leads to irreparable cellular damage and disorganization.

Inflammation

Brain death and IRI trigger the production of proinflammatory molecules and ROS, which exert a significant impact on graft performance both before and after transplantation (26, 27). The process of brain death leads to the synthesis of proinflammatory molecules (**Table 2**) during the ischemic phase. These molecules are generated by monocytes, macrophages, and neutrophils that persist in the vasculature following organ removal; endothelial cells and myocardial tissue also contribute to their production (28). Furthermore, even cardiac myocytes can synthesize cytokines during the ischemic period (29). These proinflammatory components continue to be generated during the reperfusion phase, driven by the infiltration of the graft by leukocytes and the activation of neutrophils and macrophages.

TABLE 2 Pro-inflammatory molecules produced by heart transplantation.

Family	Molecules
Cytokines	Interleukins (IL-1β, IL2, IL6, IL-10, IL-12)
	TNF-α
	IFN-y
Chemokines	MCP-1
Adhesion molecules	ICAM-1
	VECAM-1
	PECAM – 1
	Integrin
	L-selectin
Others	iNOS
	Cyclooxygenase -2

IL, interleukin; TNF-α, tumor necrosis factor alpha; IFNy, interferon gamma; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; VECAM-1, vascular cell adhesion molecule-1; PECAM–1, platelet endothelial cell adhesion molecule-1, iNOS, nitric oxide synthase inducible.

Inflammation can affect the transplanted heart graft via various mechanisms that are complex and multifaceted and could impact overall graft performance and patient outcomes. Here are some possible mechanisms and manifestations of inflammation that can negatively impact graft function: IRI, endothelial dysfunction (leading to reduced NO production, vasoconstriction, increased vascular permeability, and impaired blood flow), leukocyte infiltration (neutrophils and macrophages), myocardial edema (impairing cellular metabolism and ion channel function), allograft rejection (inflammation and immune response leading to tissue damage), fibrosis (impairing cardiac contractility and electrical conduction), cytokine storm (causing widespread tissue damage, hemodynamic instability, and multi-organ dysfunction), microvascular dysfunction (compromising oxygen delivery to cardiac cells), impaired electrical conduction (leading to arrhythmias and conduction disorders), and impaired metabolic balance.

Overall, the impact of inflammation on heart graft function is a complex interplay of immune responses, cytokine signaling, oxidative stress, and cellular interactions. Strategies aimed at mitigating inflammation, such as immunosuppressive therapies and anti-inflammatory agents, may be crucial for maintaining graft function and promoting successful HT outcomes.

Reperfusion

In the context of HT, the reperfusion phase initiates upon aortic declamping in the recipient, at which point the heart is perfused with warm blood, enabling it to restore contractility.

Vascular dysfunction and increased oxidative stress

Reperfusion involves the reinstatement of oxygenated blood supply to the tissue. During this phase, two mechanisms contribute to reperfusion injuries. Firstly, the no-reflow phenomenon arises due to inadequate microvascular reperfusion, often linked to hemostasis disorders marked by increased platelet and complement activation, as well as leukocyte aggregation. Microvascular dysfunction emerges from an imbalance between vasodilator and vasoconstrictor agents. Secondly, the sudden surge in oxygen levels gives rise to an overwhelming production of oxidative stress, surpassing the capacity of cellular antioxidant systems that were already compromised by the preceding ischemic phase. Though oxidative stress is accentuated during reperfusion, its initiation occurs during brain death and is exacerbated by ischemia. The concurrent generation of ROS and NO yields peroxynitrite ions (ONOO-), which are recognized for their cytotoxic effects, as they curtail the availability of NO, a potent vasodilator. The detrimental consequences of reintroduced oxygen also involve the opening of the mitochondrial permeability transition pore, leading to mitochondrial edema,

complete inhibition of mitochondrial functions, including ATP production, and the release of cytochrome C. This sequence of events activates the proapoptotic pathway (23). Consequently, IRI stimulates multiple cell death pathways encompassing apoptosis, necrosis, and autophagy.

Though ischemic damage predominantly results in necrosis, reperfusion injury can also trigger apoptosis (25, 30). In a rabbit model, markers of apoptosis detected in reperfused tissue were absent in normal tissue or tissue exclusively injured by ischemia (25). Other investigations have noted the emergence of apoptotic cells within the peri-necrotic region during reperfusion (31, 32).

Reperfusion and immune response

The reperfusion phase is also marked by the initiation of an innate immune response within the initial days post-transplantation, followed by an adaptive immune response that plays a role in the development of a chronic immune reaction (33, 34). Thus, in the initial week of reperfusion, the innate immune system orchestrates the recruitment and activation of monocytes, neutrophils, and dendritic cells. This process is facilitated by the generation of immunoreactive molecules during cellular stress, including damage-associated molecular patterns (DAMPs) and ligands for Toll-like receptors (TLRs) expressed within the ischemic tissue; the release of cytokines and chemokines; and the activation of endothelial cells, coupled with the expression of adhesion molecules, such as selectins.

These intricate processes intersect with the recruitment of T cells and the ensuing adaptive immune response, which occurs a few days following reperfusion and intensifies in response to the degree of graft allogenicity (35). This adaptive immune response contributes to the reduction of capillaries and leads to chronic hypoxia, ultimately contributing to delayed graft dysfunction.

Clinical correlates

Clinical presentations of IRI encompass arrhythmias, microvascular dysfunction, and myocardial stunning. Reperfusion-induced arrhythmias may be influenced by mitochondrial dysfunction. After prolonged ischemia, the mitochondria may struggle to restore or maintain their inner membrane potential, leading to destabilized action potentials and heightened vulnerability to arrhythmias (36).

Myocardial stunning denotes a temporary myocardial dysfunction that arises post-reperfusion. It is thought to stem from a combination of factors, such as oxidative stress, myocardial edema, and persistent calcium overload, even after reperfusion (37). This condition is also intertwined with microvascular injury, and enhanced regional and global myocardial function is observed when the microvasculature remains structurally intact (38–40). As myocardial stunning is reversible over time, short-term administration of inotropic agents can enhance cardiac function and organ perfusion.

Initially, the inflicted damage is reversible and, if blood flow is reinstated during this period, the structures and functions can recuperate to their normal state. However, if ischemia persists for an extended duration, the damage becomes irreparable, culminating in cell death. Before cell death is a vulnerable window during which ischemic myocytes are viable but prone to further injury upon blood flow restoration (i.e., reperfusion injury). The restoration of blood flow triggers three events that can inflict additional harm on myocytes: microvascular obstruction, deposition of red blood cells (hemorrhage), or localized inflammation that could exacerbate microcirculation damage. Myocardial edema, both intracellular and interstitial, exhibits a bimodal pattern following reperfusion. The initial surge occurs shortly after reperfusion and diminishes within a few days, whereas the second wave emerges days later and coincides with the healing process (41).

Cardioprotective strategies

Over the past few decades, numerous strategies have been proposed to mitigate IRI, capitalizing on a deeper understanding of its mechanisms and its repercussions (42). These strategies can be categorized into different groups based on their protective approach, the timing of their application, their targeted cells, and intracellular components (**Figure 2**). The principal modes of cardioprotection encompass ischemic conditioning, administration of chemical agents, and the implementation of physical interventions, such as hypothermia. These cardioprotective strategies can also be classified by when they are applied in relation to the ischemic event (i.e., prior to, during, or after) (43). Ideally, treatments that confer protection against IRI should be administered at the earliest opportunity, as the majority of cellular injuries manifest during the initial moments of reperfusion.

Static graft preservation

Hypothermia: static cold storage

SCS is the most widely employed technique for heart preservation. This method involves the prompt removal of blood from the organ, meticulous cleansing of the vascular system with a cold preservation solution and maintaining the heart in a hypothermic state of rest until it is ready for transplantation. Typically, the heart is placed within a sterile bag filled with the preservation solution, then nestled in a container equipped with ice for transportation. These measures are implemented to induce diastolic arrest in the heart, thereby curtailing its metabolic demands and mitigating the adverse impacts of ischemia during transport.

Although hypothermia does not bring cellular metabolism to a complete halt, it does decelerate the degradation of vital compounds essential for cellular viability (44). Furthermore, hypothermia curtails the rate of lysosomal organelle breakdown within cells, thereby preventing the release of autolytic enzymes and subsequent cell death. As per Vant Hoff's rule, metabolic activity at 4°C is approximately 10%–12% of the baseline observed under normothermic conditions (45). Thus, the optimal storage temperature is a controversial issue. Low temperatures

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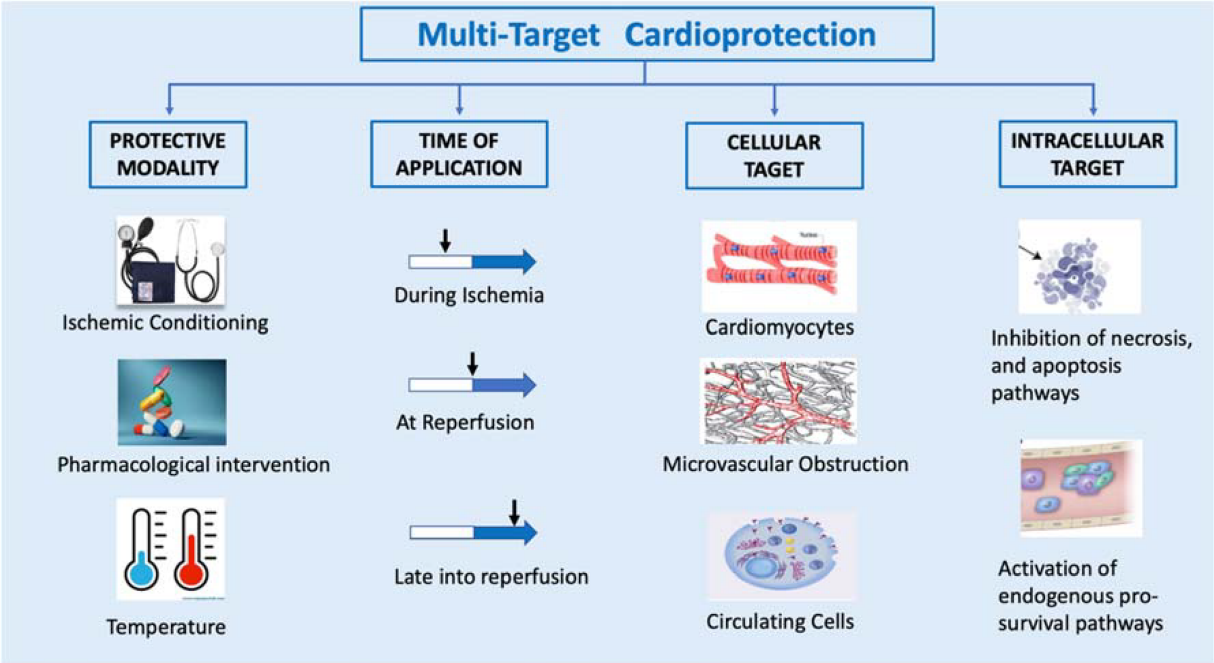

FIGURE 2
Multi-target cardioprotective strategies.

have obvious advantages in terms of energy reserve preservation but also disadvantages related to cell swelling, increased intracellular concentration of calcium, and cell membrane damage (Figure 3). Hypothermia can lead to impaired Na^+/K^+ ATPase activity, resulting in cellular swelling. Under this condition, sodium is unable to be effectively removed and,

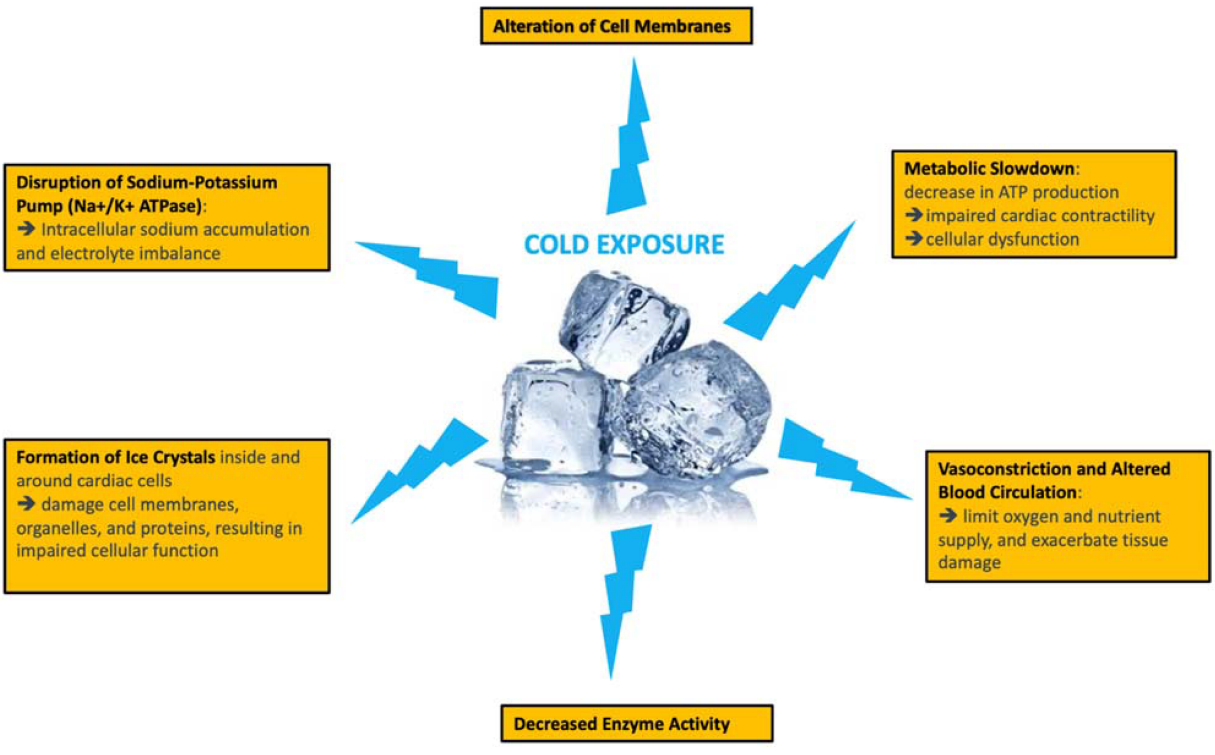

FIGURE 3
Adverse metabolic consequences of static cold storage.

instead, passively enters the cell. This creates a hyperosmolar intracellular environment, causing water to flow into the cell, resulting in cell edema. To counteract this swelling, colloids are introduced into preservation solutions.

Cold storage within the temperature range of 0–4°C triggers rapid intracellular ATP depletion. Within 4 h, nearly 95% of ATP is hydrolyzed, leading to adenosine monophosphate becoming the predominant nucleotide. This metabolic shift results in acidosis, with the production of two lactic acid molecules (46). The impact of acidosis on ischemic injury is pH-dependent. Profound acidosis activates enzymes, including phospholipases and proteases, causing damage to lysosomes and ultimately resulting in cell death. Thus, effective pH regulation is a vital role of preservative solutions.

SCS has been associated with the promotion of ROS production, likely due to mitochondrial impairment. Free or chelated iron catalyzes the formation of ROS and directly contributes to hypothermia-induced damage by causing mitochondrial dysfunction and initiating apoptosis. ROS rapidly react with other molecules, causing extensive damage to lipids, nucleic acids, and proteins (47). The ensuing mechanism of cell death seems to be dependent on ATP.

The process of rewarming can also induce harmful effects. Systemic collapse and cellular lesions may occur due to the elevation in temperature and oxygen pressure. Upon restoration of blood flow, the oxidation of hypoxanthine and xanthine produces ROS. These free radicals lead to lipid peroxidation, increased membrane permeability, oxidation of membrane proteins, and DNA damage, culminating in enzyme dysfunction. In addition, the accumulated hypoxanthine diffuses out of cells, impeding the cell's capacity to replenish its energy reserves (48, 49). These injuries may be accountable for the delayed recovery of myocardial function, acute graft failure, and long-term coronary atherosclerosis.

Paragonix Technologies (Cambridge, MA) has developed the single-use, disposable Paragonix SherpaPak® Cardiac Transport System for non-perfusing storage. Its purpose is to sustain donor heart temperatures between 4°C and 8°C for extended durations. The system involves suspending the donor heart in a preservation solution, which is de-aired to allow complete submersion of the heart to facilitate even cooling. Subsequently, the inner cannister is inserted into the outer cannister and surrounded by disposable cooling packs. These cooling packs, in contrast to regular ice that undergoes a phase change at 0°C, undergo a phase change at 5°C, thereby maintaining the desired preservation temperatures. In preclinical and clinical studies, various preservation solutions, such as Celsior, University of Wisconsin (UW), and histidine-tryptophan-ketoglutarate (HTK), have been employed with this system (50–52). Preliminary findings from an ongoing large multicenter registry involving 10 sites and 569 patients (comprising 255 ice transports and 314 SherpaPak® transports) have shown favorable early clinical outcomes in the intervention group, including reduced rates of primary graft dysfunction (PGD) and shorter stays in the intensive care unit (53). Thus, this system, which guarantees a stable temperature, provides protection against cold-related injuries, as well as real-time monitoring of data.

Ischemic conditioning

Strategies centered around ischemic conditioning encompass both preconditioning and postconditioning. The mechanisms underlying ischemic conditioning remain somewhat elusive due to their multifaceted nature. Ischemic preconditioning (IPC) serves to prevent the uncoupling of NOS and the subsequent generation of reactive oxygen and nitrogen species. In addition, it boosts signaling through proteins, such as protein kinase G, reperfusion injury rescue kinase, and survivor activation factor, in reperfused cardiomyocytes (43). IPC also seems to impact mitochondrial function (54, 55).

Preconditioning's effects can be triggered by pharmacological agents that act on identified targets and have demonstrated protective effects in animal models. Among agents that specifically open mitochondrial K-ATP channels, diazoxide has demonstrated promising outcomes, though it can induce hypotension due to its broad effects. Another potential avenue involves inducing these mechanisms through gene transfer. Gene transfer can be used to induce protective pathways akin to IPC in the context of HT. Certain genes are associated with protective mechanisms that mimic IPC. For example, genes encoding heat shock proteins (HSPs), adenosine receptors, and protein kinase C (PKC) isoforms have been identified as key players in mediating the protective effects of IPC. These genes can be transferred to the graft to induce similar protective pathways. In some cases, gene transfer can also be performed *in vivo*, where the viral vector is directly administered to the recipient after transplantation. This approach allows for targeted gene delivery to the transplanted heart. The transferred genes induce cellular responses that mimic the effects of IPC: reducing oxidative stress, improving cellular energy metabolism, promoting anti-inflammatory processes, and inhibiting apoptotic pathways. By inducing these protective pathways, the transplanted heart becomes more resistant to the detrimental effects of ischemia and reperfusion, leading to improved graft survival, reduced ischemic injury, and enhanced overall function.

However, the practical applicability of preconditioning in clinical settings remains uncertain. Effective administration of the required substances would necessitate their use prior to donor death, which is currently prohibited by ethical and legal considerations. Furthermore, the impact of such treatment would be systemic and could affect all donor organs, potentially compromising the retrieval of specific organs due to their toxicity. A possible alternative is the in-situ administration of pharmacological agents via the preservation solution to replicate the effects of preconditioning.

Heart preservation solution

To mitigate the adverse effects of IRI, a range of heart preservation solutions have been developed, each containing varying concentrations of cellular nutrients, metabolites, electrolytes, and antioxidants. The first solution, Euro Collins, was formulated in 1960, followed by the introduction of the UW

solution in 1988 (56). Subsequently, modifications and the development of new solutions have grown substantially, with more than 150 different solutions commercialized to date (57, 58). The three most commonly used solutions currently are HTK solution (Perisoc, Khöler Chemie Pharmaceuticals, Germany), UW solution (SPS-1, Poland), and Celsior solution (Institut Georges Lopez, France).

The different solutions for heart graft preservation are designed with specific compositions to optimize the viability and function of the heart during the storage period prior to transplantation. Each solution aims to maintain cellular integrity, prevent IRI, and support the necessary metabolic processes for effective recovery post-transplantation. The main reasons underlying the distinct compositions of heart graft preservation solutions include:

- *Nutrient and electrolyte provision*: Preservation solutions must contain essential nutrients, such as carbohydrates, amino acids, and electrolytes to sustain fundamental metabolic processes and maintain electrolyte balance in cardiac cells during the storage period.
- *Oxygenation and antioxidants*: Preservation solutions may incorporate antioxidant agents to shield cardiac cells from damage caused by free radicals produced during ischemia-reperfusion. Adequate oxygenation in the solution can also support aerobic metabolism of cells.
- *Acid-base balance and pH maintenance*: Preservation solutions need to uphold acid-base equilibrium and appropriate pH levels to prevent metabolic acidosis that can occur during ischemia.
- *Cellular edema reduction*: Some solutions contain osmotic agents to minimize water accumulation in cardiac cells, reducing the risk of cellular edema and structural damage.
- *Inhibition of inflammatory responses and apoptosis*: Certain solutions may include anti-inflammatory agents or substances that inhibit apoptotic pathways to mitigate cellular damage induced by ischemia-reperfusion.
- *Support of mitochondrial function*: Mitochondria are critical for ATP production and cell survival. Some solutions may contain energy substrates and cofactors to support mitochondrial function.
- *Prevention of endothelial injury*: Preservation solutions aim to preserve vascular endothelial integrity to promote adequate blood flow and minimize the risks of thrombosis or endothelial dysfunction.

Ultimately, the compositions of heart graft preservation solutions aim to optimize cellular viability and cardiac function during the storage period to mitigate ischemia-reperfusion-related damage and maximize the success of HT. Each solution may offer specific advantages based on its composition, route of administration, and capacity to protect the heart against various consequences of ischemia and reperfusion.

Numerous studies have attempted to compare post-transplantation outcomes associated with various preservation solutions. However, the results have been inconsistent (59) and have not clearly established the superiority of one solution over another (60–63). In a larger observational and retrospective study, UW solution ($n = 3{,}107$) demonstrated a significantly higher 1-year survival rate than Celsior solution ($n = 1{,}803$), though this difference may not have clinical relevance (89.6% vs. 87.0%, $p < 0.01$) (64). Comparative analyses of different preservation solutions are strongly influenced by confounding factors related to extended criteria for organ donation, duration of cold ischemia, complex recipient surgical conditions (e.g., redo surgery and mechanical circulatory support), and the effects of IRI.

Regardless of the preservation solution used, optimal results with SCS are achieved when the ischemia time remains under 4 h. Each additional hour of ischemia beyond 4 h amplifies the risk of graft failure by 43% and increases the 1-year post-transplant mortality risk by 25% (21).

Additive drugs during preservation

Targeting cellular signaling pathways that contribute to IRI outcomes is promising as a therapeutic approach. Numerous potential therapies are being explored. Antioxidants and NO transport agents are of particular interest due to the pivotal role of oxidative stress and endothelial damage (48, 49). In addition, drugs that reduce graft immunogenicity before transplantation could help mitigate post-transplantation damage. An alternative approach involves incorporating valproic acid into perfusate, which has had encouraging results (65, 66). The application of metabolomics, proteomics, and genomics techniques to organ preservation offers a promising avenue for identifying new markers of IRI-related damage and subsequent development of novel therapeutic targets.

Anti-inflammatory therapies have also emerged as intriguing targets, though they have been comparatively underexplored in this context. Colchicine, which exerts diverse anti-inflammatory effects by inhibiting neutrophil chemoattraction, the inflammasome network, and pro-inflammatory cytokines, stands out as a potential candidate (67). By disrupting tubulin, colchicine interferes with multiple inflammatory pathways, leading to diminished neutrophil function and reduced migration across the vascular endothelium. Proinflammatory cytokines and adhesion molecule expression are also potential targets for colchicine, thereby impeding local production of coronary chemokines, such as MCP-1 (68), and the secretion of tumor necrosis factor-α by macrophages. Colchicine may even exhibit some anti-fibrotic effects. Various animal studies investigating its cardioprotective effects have found that lower doses of colchicine can inhibit heart apoptosis in rat models (69) and generate anti-fibrotic effects *in vitro* (70), potentially through microtubule cytoskeleton remodeling and/or direct anti-inflammatory actions. Its anti-inflammatory effects are characterized by a reduction in cytokines involved in the post-ischemic inflammatory response, including IL-6, MCP-1, and IL-10 (71, 72).

Dynamic graft preservation

Ex situ machine perfusion

Ex situ machine perfusion was initially proposed to prolong the duration of organ preservation, with the added benefit of

potentially expanding the donor pool and improving utilization rates. By supplying the metabolic needs of the myocardium, perfusion can effectively minimize irreversible ischemic cell injury and subsequent cell death. Various heart perfusion systems, categorized as either hypothermic machine perfusion (HMP) or ex situ normothermic perfusion (ESNP), have demonstrated successful preservation of animal and/or human hearts (73). Notably, the longest reported successful human heart preservation time, 16 h, was achieved using ESNP (74).

These perfusion devices are designed to be portable and enable an extended and secure preservation window, facilitating the use of hearts from donors located at a distance. Currently, a single commercially available perfusion system is available for clinical applications, known as the Organ Care System® (OCS, TransMedics, Andovers, USA) (ESNP). In Europe, a multicenter randomized clinical trial is currently underway to explore the safety and potential advantages of the XVivo® technology for heart preservation (XVivo Perfusion AB, Göteborg, Sweden) (HMP) (NCT03991923) (75, 76). The clinical significance of these devices is broad, encompassing an expansion of preservation duration, assessment of extended-criteria donors, and the utilization of re-perfused donor hearts retrieved following controlled circulatory death. Detailed summaries of studies with robust methodologies investigating ex situ machine perfusion are provided in **Table 3**.

Ex situ normothermic perfusion

OCS configuration

Following the retrieval of the heart graft previously preserved using a hypothermic solution, the graft is prepared and positioned on a dedicated module designed for isolated perfusion (see **Supplementary Figure S1**). The OCS perfusion system consists of a reservoir and an oxygenator, both connected to a centrifugal pump. An aortic cannula is affixed to the graft's aorta and is responsible for perfusing the aortic root and coronary arteries with nutrient-enriched donor blood. An additional cannula is placed in the pulmonary artery to collect blood returning from the coronary sinus, enabling the measurement of blood lactate, which is the only available predictive factor (modest sensitivity and specificity) of myocardial viability to date and, consequently, of the quality of ex situ perfusion (74–77). Lastly, a third cannula is introduced into the left atrium to relieve pressure in the left ventricle.

The anterograde coronary perfusion is regulated by maintaining a minimum pressure of 40 mmHg to ensure sufficient coronary flow and avoid inadequate perfusion at lower pressures (75–77). Pseudo-pulsatility is induced through external pacing of myocardial contractions at a rate of 80 beats per minute. This stimulation aids in emptying the right ventricle, facilitating the collection of coronary venous return while maintaining the capillary bed open and enhancing extracorporeal perfusion (77). A drainage cannula is inserted via the mitral valve to prevent coronary gas embolisms and to offer passive unloading of the left ventricle during preservation.

After the arterial shunt is activated, the heart is gently massaged during the rewarming process. The heart may regain its spontaneous rhythm or fibrillate, necessitating an external defibrillator shock. When electrical activity resumes, the inferior vena cava is tied off. Gradually, the assisted flow is increased to reach a target range of 650–850 ml/min measured at the return to the pulmonary artery, indicating the coronary flow. This is achieved to maintain a desired mean arterial pressure of

TABLE 3 Complete or recruiting studies investigating *ex vivo* machine perfusion for heart transplantation.

NCT	Type of device	Location	*n*	Protocol	Primary endpoint
NCT03991923	XVIVO	Europe (15 centers)	202	RCT XVIVO heart preservation devices VS cold static storage	30 days mortality and 30 days graft dysfunction
NCT04066127	NIHP	Sweden	66	RCT Non-ischemic heart preservation (NIHP) VS Standard ischemic cold static storage	Survival free of acute cellular rejection and re-transplantation
NCT03831048	OCS	United States	180	RCT OCS heart System VS Cold Storage	Patient survival 6 months post-transplant
NCT03835754	OCS	United States	75	Observational prospective study Single Group Assignment OCS heart System	Patient survival 30 days post transplant
NCT03150147	NIHP	Sweden	47	RCT NIHP VS Ischemic cold static storage	Composite endpoint of patient death due to graft failure, re-transplantation due to graft failure, severe primary graft dysfunction (PGD), need for extra corporal mechanical support such as ECMO within 7 days post transplantation, or acute cellular rejection (ACR) ≥grade 2.
NCT05741723	OCS	United States	276	Observational prospective study Single Group Assignment OCS heart System	Patient survival through 5 years post-transplant
NCT00855712	OCS	United States	128	RCT Organ Care System VS cold static storage	30-day patient survival following transplantation with the originally transplanted heart and no mechanical circulatory assist device at day 30
NCT05047068	OCS	United States	350	Observational prospective study Single Group Assignment OCS heart System	Patient survival at one-year post- heart transplant.

ECMO: extracorporeal membrane oxygenation; OCS: organ care system; RCT: randomized controlled trial.

70–85 mmHg. Adjustments are made to the mean aortic pressure and coronary flow to ensure the extraction of myocardial lactate, which is evident by a positive arterio-venous difference.

Clinical use and applications

Several prospective non-randomized trials comparing SCS with the use of the OCS® demonstrated similar survival rates at 30 days, 1 year, and 2 years (76, 78, 79). The implementation of OCS was associated with extended overall preservation times, reduced cold ischemia duration, and a trend toward decreased PGF rates. In addition to preserving standard donor grafts, clinical interest has been directed towards employing OCS® for marginal donors or cases with long anticipated preservation times. This approach aims to overcome the constraints of the current limited donor pool. The EXPAND trial (80) encompassed grafts with an expected total ischemic time of either ≥4 h or >2 h combined with specific criteria, such as left ventricular hypertrophy, ejection fraction of 40%–50%, circulatory arrest time ≥20 min, or age exceeding 55 years. Eighty-one percent of the included grafts were utilized, resulting in 30-day and 6-month survival rates of 94.7% and 88.0%, respectively. Severe early graft dysfunction within the initial 24 h was observed in 10.7% of cases. Nevertheless, the absence of a comparative group somewhat limits the robustness of these findings and their applicability in clinical practice. Various institutions have independently reported their experiences utilizing OCS® for expanded criteria donors (81). Certain studies have described marginal donor series that were transplanted following OCS preservation, with an average total preservation time of 284 min. In these cases, the 1-year survival rate reached 96.2% (82).

Hypothermic machine perfusion (HMP): X VIVO NIHP technology

Initial research efforts in the field of machine heart perfusion concentrated on hypothermic oxygenated perfusion methods (83). Numerous preclinical studies investigating hypothermic oxygenated perfusion have demonstrated the successful preservation of hearts for periods of up to 48 h (84, 85). The XVIVO® Heart Preservation System (XVIVO Inc, Gothenburg, Sweden) (**Supplementary Figure 2**) is currently undergoing testing in a phase II clinical trial. In contrast to warm graft reperfusion, HMP hinges on the utilization of cardioplegia and hypothermia to induce heart arrest, thereby minimizing metabolic demand during perfusion (75, 86). During this process, the heart is subjected to perfusion with a cold solution (8°C) that is enriched with nutrients and hormones. This cardioplegia solution also includes red blood cells, which serve to restore the depleted ATP levels within myocardial cells, as supported by preclinical studies (87–89).

HMP configuration

The HMP procedure involves the use of a serial roller pump, an oxygenator, a leukocyte filter, and a heater/cooler component. To sustain the perfusion circuit, 2.5 L of perfusion solution and 500 ml of compatible irradiated and leuko-reduced donor/recipient blood are required, with a target hematocrit of 18%. The heart is connected to the HMP system using an aortic cannula. Once the heart is in position, the ex-situ circulation process is initiated. Oxygenated blood is introduced into the aortic root, aiming for a perfusion pressure of 20 mmHg and a coronary flow rate of 150–250 milliliters per minute. The heater/cooler mechanism maintains the temperature at 8°C and the pH at 7.4.

Clinical use and applications

HMP studies in the context of HT are limited, but they have shown promising results, particularly in the field of lung preservation and evaluation of expanded criteria lung transplant donors. Early clinical applications of the HMP system have also yielded positive outcomes for donor heart preservation. In a nonrandomized open-label phase 2 trial (75), 6-month mortality was lower for patients who underwent HMP compared to those who underwent SCS. The SCS group experienced four deaths within 6 months after transplantation and three cardiac-related adverse events, whereas the HMP group reported no deaths or cardiac-related adverse events. The median preservation times were 223 min for HMP and 194 min for SCS. Importantly, no significant PGF cases were reported. This trial marked the first-in-human study demonstrating the feasibility and safety of normothermic isolated heart perfusion (NIHP) for clinical HT.

Overall, though HMP studies in HT are relatively limited, the outcomes observed thus far are promising and suggest that HMP could play a crucial role in improving donor heart preservation and transplantation outcomes.

Ex situ machine perfusion limitations

However advantageous these techniques may be, they are not devoid of substantial limitations. In addition to their high cost and the need for specialized transportation, these methods also demand intricate technical expertise for graft retrieval, device implementation, and oversight during transit as outlined in **Table 4**. Furthermore, the process of ex situ normothermic perfusion (ESNP) introduces successive bouts of ischemia-reperfusion during organ harvesting and transplantation, potentially heightening the risk of cellular and myocardial damage.

The current assessment of graft performance during ESNP is confined to observing lactate kinetics and clearance within a perfused, beating heart devoid of afterload conditions. This evaluation approach restricts the comprehensive appraisal of heart function, limiting it to a Langendorf perfusion model, which falls short in terms of sensitivity and specificity. Critical aspects, such as systolic and diastolic functions, cannot be accurately gauged in the absence of preload and afterload considerations, constraining the scope of graft evaluation.

Preclinical studies are imperative to comprehensively understanding the intricate dynamics of IRI across different levels, ranging from the tissue to the microcirculation and endothelium, as well as cellular calcium homeostasis. For example, a porcine model demonstrated a correlation between lactate kinetics and the success or failure of normothermic ex situ preservation, as determined by left ventricular contractility measurements (90). In another study, the normothermic ex situ

TABLE 4 Comparison of different techniques for heart graft preservation.

	Static cold storage	Transmedics organ care system	XVIVO perfusion XHPS
Price	800–1,000 euros (preservation solution)	30–35,000 euros	Not available
Weight	2.5 Kg	45 Kg	25 Kg
Ergonomics	+++	+	++
Storage temperature	4°C	34–36°C	8°C
Storage duration	4 h	Until 4–6 h	Until 24–48 h
Storage solution	Static	Dynamic	Dynamic
	• 2 L of preservation solution	• 1.5 L of donor leukoreduced blood	• 2.5 L of perfusion solution
		• +500 ml of perfusion solution	• +500 ml of compatible donor/recipient blood irradiated and leukoreduced
Perfusion pressure	–	70–85 mmhg	20 mmhg
Coronary flow	–	650–850 ml/min	150–250 ml/min
Advantages	• Safe organ storage	• Monitor organ function (aortic pressure, coronary flow, heart rate, blood temperature)	• Uses preservation solution
	• Uses preservation solution.		• Monitor pO2 and pH of perfusate
	• Cheap		
	• Homogeneous temperature		• Allows x-ray
	• Immersed graft	• Housing enables ultrasound assessment and blood sampling	• Console is reusable but Perfusion set is once use
	• Easy to use		
	• No electrical power required		
	• Handling	• Console is reusable but Perfusion set is one time use	• Proven superiority
	• Energy reserves preservation		
Disadvantages	• Ischemic reperfusion injury	• Expensive	• Expensive
	• Short Storage duration	• Needs more staff and qualified personnel	• Needs more staff and qualified personnel
	• Cell swelling	• No functional working mode	
	• Increased Ca2+	• No proven superiority	
	• Damage to cell membranes.	• Successive periods of ischemia-reperfusion	

model exhibited superiority over hypothermic crystalloid solution preservation, demonstrating protective effects by inhibiting apoptosis and oxidative stress in coronary arteries, enhancing both endothelium-independent and endothelium-dependent vasorelaxation, and promoting antioxidant production to prevent ONOO-free radical formation. This approach also resulted in increased ICAM-1 expression in grafts (91). Paradoxically, when comparing three preservation methods after a 30-minute delay in warm ischemia (including warm oxygenated blood, 4°C crystalloid HTK, and a novel HTK-N solute at 4°C), contractility indices were more favorable in the HTK-N and HTK groups compared to the warm blood perfusion group. In addition, immunohistochemical markers, immunoreactivity, and cellular edema were notably reduced in the HTK-N group compared to the warm reperfusion group (92).

Given these complexities, there is a pressing need for additional comparative data to elucidate the inflammatory and immunohistochemical phenomena between ex vivo normothermic perfusion (ESNP) and the conventional cold static preservation approach. Such data could provide a more comprehensive understanding of the underlying processes and aid in refining these preservation techniques.

Perspectives

Ex situ therapeutic interventions

Ex situ perfusion systems present a significant opportunity to serve as a prominent conduit for the administration of therapeutic interventions to donor hearts without causing adverse effects on other donor or recipient organs. The central objective of these interventions is to mitigate the impact of IRI, with a particular focus on the potential to rejuvenate marginal organs for viable transplantation. Although these therapeutic applications are still in the preclinical phase and have not yet undergone clinical testing, their preliminary outcomes are promising. The prospective integration of ex situ therapies has

the potential to not only enhance graft viability, but also contribute to expanding the existing donor organ pool, a prospect that has significant potential for the field of transplantation.

There are several potential therapeutic strategies that can be explored within the ex-situ perfusion context:

- **Pharmacological agents**: Various pharmacological agents, such as antioxidants, anti-inflammatory drugs, and vasodilators, can be administered to the donor heart during ex situ perfusion. These agents aim to counteract oxidative stress, reduce inflammation, and improve vascular function, ultimately protecting the heart from IRI-related damage.
- **Gene therapy**: Gene transfer techniques can be utilized to introduce specific genes into the donor heart during ex situ perfusion. These genes can encode for protective factors or enzymes that counteract IRI processes. For example, introducing genes that promote antioxidant production or inhibit apoptosis could enhance the heart's resistance to IRI.
- **Cell-based therapies:** Ex situ perfusion systems can facilitate the delivery of therapeutic cells, such as stem cells or genetically modified cells, directly to the donor heart. These cells have the potential to promote tissue repair, regeneration, and immunomodulation, further enhancing the heart's viability.
- **Metabolic modulation:** Manipulating the metabolic pathways within the donor heart during ex situ perfusion could promote energy production and reduce the negative consequences of metabolic disruption during IRI.
- **Targeted drug delivery:** Nanoparticles and other drug delivery systems can be designed to target specific cellular pathways and deliver therapeutic compounds directly to the heart tissue, minimizing systemic effects and maximizing efficacy.

Though these strategies have shown great promise in preclinical studies, it is important to note that translating them into clinical practice requires rigorous testing and validation. Clinical trials are needed to assess the safety, efficacy, and long-term outcomes of these interventions in human donor hearts. If successful, ex situ perfusion-based interventions could revolutionize the field of organ transplantation by expanding the pool of viable donor organs, reducing the incidence of IRI-related complications, and improving overall graft quality.

Blocking apoptosis and necrosis pathways

Interception of the apoptosis pathway in porcine hearts using hypothermic perfusion solutions infused with small interfering RNA molecules targeting key apoptotic and inflammatory enzymes has had notable outcomes in diminishing cellular apoptosis and mitigating myocyte injury. Furthermore, this approach has resulted in enhancement of donor myocardial function (93–95). In a distinct porcine transplantation model, the introduction of oxygen-derived free radical scavengers into the perfusion process has shown the potential to enhance graft functionality while concurrently ameliorating cellular edema (54).

Yet another preclinical investigation unveiled the efficacy of a therapeutic regimen that couples two inhibitors of the necrosis pathway, resulting in a discernible reduction in IRI among rat cardiomyocytes (95, 96). Therapies that foster angiogenesis have also garnered attention. Examples encompass interventions involving the inclusion of vascular endothelial growth factor (VEGF) (97), prokinectin receptor-1 (98), or human multipotent stromal cells (98, 99). These interventions have manifested the capacity to elevate the survival rate of cardiomyocytes subjected to ischemia.

Extracellular vesicle therapy

Extracellular vesicles (EVs) derived from various cell lines have exhibited a range of beneficial properties in both *in vitro* and *in vivo* studies, including immunomodulation, antioxidant activity, anti-inflammatory effects, and tissue repair capabilities. These EVs possess the ability to enter target cells and modulate signaling pathways that contribute to tissue healing. EVs have also demonstrated the capacity to hinder left ventricular dilatation and enhance cardiac contractility. At the cellular level, EVs have been effective in mitigating energy depletion, reducing oxidative stress, and curtailing the infiltration of neutrophils and macrophages by activating the protein kinase B and glycogen synthase kinase 3 (AKT/GSK3) pathways. Various animal models have revealed that the administration of EVs prior to, during, or after IRI can counteract inflammatory responses, diminish infarct size, and promote reparative processes (100) while operating independently of circulating cells such as leukocytes and platelets. The use of EVs originating from human lymphoid T cell lines to deliver Sonic hedgehog (SHH) has also demonstrated therapeutic efficacy (101).

In the context of myocardial IRI in murine models, EVs derived from cardiac progenitor cells (CPCs) have demonstrated significant cardioprotective effects (102). When administered intramyocardially following IRI, CPC-derived EVs effectively restrain oxidative stress and myocardial apoptosis by transferring protective mRNA molecules, suggesting that these EVs exert their cardioprotective influence by influencing cellular signaling pathways (103). Collectively, these investigations underscore the potential of diverse EV types in safeguarding the heart from IRI. In the realm of HT, the timing of EV administration offers flexibility, including options such as before ischemia (aortic clamping), during organ preservation (ex situ perfusion), before reperfusion, or post-organ reperfusion. Despite the challenges inherent in this field, several clinical trials are anticipated in the upcoming years to evaluate the therapeutic promise of EVs in patients undergoing solid organ IRI.

Donor heart-specific gene therapy

Some studies employing a porcine model of HT with normothermic ex situ perfusion have delved into the utilization of adenoviral vectors and adeno-associated viral vectors for gene administration using the OCS® system (104, 105). The investigations aimed to ascertain the feasibility of employing ex situ perfusion as a conduit for delivering a viral vector to a donor heart during storage, with a focus on evaluating the resultant biodistribution and expression levels of the transgene in

the recipient post-transplant. The impact of various components within the OCS®, such as the proprietary solution, donor blood, and ex situ circuitry tubing and oxygenators, was scrutinized. A refined ex situ perfusion strategy optimized for efficient adenoviral vector transduction was employed to introduce 5×10^{13} total viral particles of an adenoviral firefly luciferase vector harboring a cytomegalovirus promoter into porcine donor hearts before heterotopic implantation. The comprehensive assessment encompassed levels of expression, protein activity, and dispersion of the firefly luciferase protein, and was conducted over a series of three heart transplants with a post-transplant endpoint of 5 days. Notably, though the perfusion solution and ex situ circuitry had no influence on viral vector transduction, the serum or plasma components of the donor blood significantly hindered the transduction process (104).

Another study within this domain explored the administration of recombinant adeno-associated viral vectors (rAAVs) during normothermic ex situ perfusion to facilitate transgene delivery to porcine cardiac allografts. A myocardially enhanced variant known as SASTG was utilized to assess the transduction efficiency in OCS perfusate. Employing normothermic ex situ perfusion, the introduction of SASTGs containing the firefly luciferase transgene into porcine donor hearts was executed across four heterotopic transplantation procedures. This approach yielded sustained, dose-dependent transgene expression in allografts within 30 days, showing no indications of off-target transgene expression. This investigation underscores the feasibility and efficacy of using AAV vectors during ex situ perfusion to achieve gene delivery to a large animal allograft (105).

Subsequent experiments pertaining to gene delivery to the explanted pig heart involved a method of blood washing prior to the secondary infusion of blood into the perfusate, thereby removing undesired plasma or serum components from the donor blood before its incorporation into the circuit. The enzymatic assessment of luciferase activity within tissues (native heart, allograft, liver, etc.) obtained on days 5 (104) and 30 (103) post-transplant unveiled robust and widespread luciferase activity throughout all sections of the allograft (right and left atria, right and left ventricles, coronary arteries), in contrast to the recipient's native organs. Crucially, luciferase activity in the recipient's heart, liver, lung, spleen, or psoas muscle remained within baseline limits. Luciferase protein expression in the allograft exhibited uniformity and vigor across myocardial areas and coronary arteries.

Taken together, these two investigations collectively establish the feasibility of employing two distinct types of viral vectors to achieve potent and comprehensive transgene expression through administration utilizing the OCS®. This innovative approach to viral vector delivery introduces the prospect of biological modification of the allograft prior to implantation, which could potentially enhance post-transplant outcomes.

Donation after circulatory death

As the demand for donor hearts continues to rise, transplant centers are increasingly considering the utilization of expanded-criteria donors. This resurgence in interest also extends to the population of donation after circulatory death (DCD), which has the potential to substantially augment the pool of available transplants. Furthermore, DCD can offer grafts from donors who typically possess lower comorbidity levels and are often younger (106). Nonetheless, a primary criticism of this approach revolves around the necessity for the heart to undergo cardiac arrest upon withdrawal of life support, leading to ischemic injury. Several strategies are currently employed and have been investigated, either involving or not involving initial regional normothermic perfusion (NRP), followed by subsequent SCS or normothermic reperfusion using the OCS® during transport prior to transplantation. Nonetheless, a universal consensus regarding the acceptability of NRP has not yet been reached across various countries and transplantation centers.

Encouraging outcomes are also being observed with direct harvesting of DCD hearts combined with ex situ reperfusion.

A recent preclinical study (107) utilizing a porcine model compared three different strategies for heart preservation after circulatory death: (1) normothermic regional perfusion combined with SCS, (2) normothermic regional perfusion combined with oxygenated HMP (X-VIVO® system), or (3) direct procurement combined with oxygenated HMP. Following preservation, HT was performed. The study found that only hearts transplanted in the HMP groups exhibited a significant increase in biventricular contractility 2 h after cardiopulmonary bypass, requiring significantly less dobutamine to maintain cardiac output than in the SCS group. These results provided strong support for the potential applicability of the HMP device in human HT.

Messer and colleagues shared their 5-year experience involving DCD donation, encompassing 79 transplants derived from DCD donors (22 with NRP and 57 with direct harvesting using OCS®). This approach notably amplified the transplant activity of the center by an impressive 48% (164 transplants in total). Survival rates at 30 days [DCD: 97% vs. brainstem death (BSD): 99%; $p = 1.00$] and 1 year (DCD: 91% vs. BSD: 89%, $p = 0.72$) were comparable between the two groups. The US Food and Drug Administration granted approval for application of the OCS® in heart preservation after DCD donation in April 2022, and the results of a dedicated clinical trial (NCT03831048) are anticipated.

In a recent randomized non-inferiority trial, the 6-month survival following transplantation with a resuscitated donor heart assessed via non-ischemic extracorporeal perfusion after circulatory death was on par with survival rates after conventional transplantation involving donor hearts preserved using cold storage after brain death. Notably, 6-month survival was 94% among recipients of a heart from a circulatory-death donor, compared to 90% among recipients of a heart from a brain-death donor (least-squares mean difference, −3 percentage points; 90% CI, −10 to 3; $p < 0.001$ for noninferiority) (81).

Early outcomes stemming from DCD donation have been promising and present a potential avenue for tapping into an underutilized donor pool. Current projections suggest that widespread adoption of DCD donation could elevate the annual count of adult heart transplants in the US alone by 300–600 (108). Given that this avenue has only recently been re-explored, the long-term performance of DCD grafts remains relatively uncharted territory.

Conclusion

Transplantation stands as the most efficacious approach to addressing end-stage heart disease, yet the insatiable demand for donor organs is anticipated to persistently outpace the available supply. The success of HT hinges upon the preservation of grafts against the detrimental effects of IRI. Capitalizing on the ongoing advancements in techniques and technologies, transplant centers are actively seeking to enlarge the donor pool by pushing the boundaries of established practices and constraints on ischemia time. In this pursuit, the evolution of ex situ perfusion machines has emerged as a pivotal development. These systems offer a dual advantage of enhanced preservation and more precise assessment of organs procured from expanded criteria and donation after circulatory death. Furthermore, the versatility of these ex-situ perfusion platforms extends to their potential as conduits for targeted therapies directed at donor organs prior to transplantation. As the landscape of HT continues to evolve, these innovations promise to redefine the limits of graft viability and optimize outcomes for both recipients and donors.

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Coordination and supervision: AU and CD. Funding acquisition: PB, CD, and FR. Investigations: all authors. Writing-original draft: AU and CD. Writing-review and editing: AU, FR, AM, AL, and CD. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Mitochondrial transplantation: the advance to therapeutic application and molecular modulation

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Mitochondrial transplantation provides a novel methodology for rescue of cell viability and cell function following ischemia-reperfusion injury and applications for other pathologies are expanding. In this review we present our methods and acquired data and evidence accumulated to support the use of mitochondrial transplantation.

KEYWORDS

mitochondria, mitochondrial transplantation, heart, transcriptomics, proteomics

1. Introduction

The heart is an obligate aerobic organ and is dependent upon oxygen delivery to the mitochondria to ensure function. Mitochondria represent approximately 30% of cardiomyocyte cell and extract >75% of coronary arterial oxygen just to meet homeostatic requirements (1). Increased functional demands are therefore dependent on increased oxygen delivery either through increased coronary blood flow and/or oxygen extraction.

Interruption or limitation in coronary blood flow limits oxygen delivery to the myocardium such that it is no longer sufficient to meet metabolic demands and results in myocardial ischemia. It is generally accepted that the limitation or cessation of coronary blood flow to the myocardium is the initial step in the myriad of processes leading to myocardial ischemia injury and these processes primarily effect the mitochondria (2–4).

With the onset of myocardial ischemia, alterations occur in mitochondrial structure and function that negatively impact mitochondrial function (5–8). The mitochondria become swollen and there is cristae disruption and mitochondrial calcium accumulation and the electron transport chain complexes I-V show decreased activity leading to decreased high energy phosphate synthesis that is needed to maintain cellular function. Mitochondrial DNA is also damaged leading to decreased mitochondrial transcriptomics, proteomics and metabolomics and the intrinsic apoptotic pathway is activated, leading to loss of cellular viability (6, 8–11). Accompanying these changes are alterations in transcriptomic, proteomic and metabolomic pathway regulation that are directly associated with mitochondrial and contractile function (12–15). All these events occur during ischemia and despite the restoration of coronary blood flow and oxygen delivery to the myocardium, they persist during reperfusion to significantly compromise myocardial cellular viability and function (8–10).

Interventions to limit mitochondrial dysfunction during ischemia and reperfusion have mainly been directed to mechanisms or pathways up- or down-stream of the mitochondrion. While somewhat efficacious, these interventions have provided only minimal clinical utility for the amelioration of the effects of ischemia-reperfusion injury. As an alternative we have proposed organelle transplant, mitochondrial transplantation, to directly address mitochondrial dysfunction. Mitochondrial transplantation is premised on the observed

alterations in mitochondrial function that manifest during ischemia and persist through reperfusion. We hypothesized that the replacement or augmentation of damaged mitochondria through the transplantation of viable, respiration competent mitochondria, isolated from non-ischemic tissue, and then delivered to the ischemic organ would enhance post-ischemic myocardial functional recovery and myocellular viability (14).

Mitochondrial transplantation provides a novel methodology for the rescue of cell viability and cell function following ischemia-reperfusion injury and applications for other pathologies are expanding. A systematic review of animal and human studies supports the beneficial effects of mitochondrial transplantation for the amelioration of ischemia- reperfusion injury (16). In this review we present our methods and acquired data and the accumulated evidence to support the use of mitochondrial transplantation.

2. Mitochondrial uptake and functional integration

The earliest example of naked mitochondrial uptake into cells was reported by Clark and Shay (17). The authors used simple coincubation of isolated mitochondria from antibiotic resistant cells with antibiotic sensitivity to show that antibiotic resistance could be transferred. The authors showed that the antibiotic resistant mitochondria were taken up by the antibiotic sensitive cells by endocytosis and that the transferred mitochondria were functional and conferred antibiotic resistance. These early studies, termed mitochondrial transformation by the authors, were mostly observational and were posited as a novel means for studying mitochondrial genetics in mammalian cells and provided early seminal evidence for the uptake and functional integration of exogenous mitochondria.

The uptake of mitochondria into cells has been demonstrated by numerous authors using a variety of methods (18–23). Katrangi et al. (18) showed that co-incubation of isolated mitochondria from human mesenchymal stem cells (hMSC) with non-respiration functional A5490 p^0 cells having fully depleted mtDNA, rescued cell function and restored cellular respiration. Uptake was confirmed by fluorescent labelling and PCR analysis.

Kitani et al. (19) used DsRed2 mitochondria isolated from human uterine EMCs-DsRed2 cells to demonstrate uptake and functional integration of mitochondria into recipient H9c2 cells, stably expressing green fluorescent protein (GFP). The authors used co-incubation and showed the engulfment of exogenous mitochondria. The exogenous mitochondria were evident in the perinuclear space inside the recipient cells within 1–2 h. The transferred mitochondria were able to rescue the mitochondrial respiratory function and improved the cellular viability in mitochondrial DNA-depleted cells and these effects lasted six days.

Pacak et al. (20) also showed that co-incubation of naked mitochondria with non-functional HeLa p^o cells lacking mitochondrial DNA, resulted in the rapid uptake of the exogenous mitochondria. Uptake was visualized using mitochondria labelled with pHrodo a label that specifically detects phagocytosis and endocytosis with a pH-sensitive fluorogenic dye that is non-fluorescent at neutral pH and bright red upon acidification inside the cytosol. The internalized mitochondria rescued mitochondrial oxygen uptake and ATP synthesis and replaced mitochondrial DNA. These effects were present for 53 cell divisions over 23 days.

Cowan et al. (24) used three-dimensional super-resolution structured illumination microscopy (3-D SR-SIM) and transmission electron microscopy (TEM) to reveal the intracellular position of endocytosed mitochondria in human induced pluripotent stem cell-derived cardiomyocytes and human cardiac fibroblasts. These studies used a human cardiac fibroblast cell line as the source of mitochondria for transplantation and a human iPS cardiomyocyte cell line as the recipient cell line to demonstrate the uptake and functional integration of mitochondria into human cells.

Cowan et al. (24) used distinct fluorescent labeling of human induced pluripotent stem cell-derived cardiomyocytes and human cardiac fibroblasts using baculovirus-mediated transfer of mammalian fusion genes containing fluorescent probes (green; GFP or red; RFP) fused to the leader sequence of E1 alpha pyruvate dehydrogenase. Pyruvate dehydrogenase is a matrix associated protein and fusion labeling provides a reliable methodology for imaging and detection. The authors labeled mitochondria in human induced pluripotent stem cell-derived cardiomyocytes with RFP while mitochondria in human cardiac fibroblasts were labeled with GFP.

Cowan et al. (24) co-incubated the GFP labeled mitochondria, isolated from human cardiac fibroblasts with human induced pluripotent stem cell-derived cardiomyocytes containing RFP labeled mitochondria. These experiments were replicated by coincubation of human induced pluripotent stem cell-derived cardiomyocytes with mitochondria isolated from human cardiac fibroblasts labelled with gold nanoparticles with subsequent TEM analysis.

Cowan et al. (24) showed that mitochondrial uptake into cells is rapid and can be seen at 2.5 min post-delivery, the shortest time frame allowable for experimental determination. The authors showed that the transplanted mitochondria were detected adjacent to the apical cell surface, undergoing endocytosis and then being taken up and released from early and late endosomes and then fusing with intrinsic mitochondria within the cell. The transplanted mitochondria were of the proper size and shape and contained the mitochondrial fusion proteins MFN1, MFN2 and OPA1. A small amount of DRP1 was detected but was not phosphorylated, suggesting fission did not occur. Greater than 80% of the transplanted mitochondria could be detected in association with early endosomes and late endosomes and then released into the cell to fuse with the endogenous mitochondria. Greater than 70% of the endocytosed mitochondria co-localized and fused with endogenous mitochondria.

Kesner et al. (21) have also shown mitochondrial uptake into cells. In these studies mitochondria labelled with DsRed were co-incubated with HepG2 cells where they fused with the intrinsic mitochondria. The authors reported that internalization of exogenous mitochondria can occur in as little as 10 min and

showed that uptake of the exogenous mitochondria lasted for at least 6 days. Further experiments using patient cells showed the transplanted mitochondria increased cell viability and mitochondrial activity.

These studies have been recently confirmed by Rossi et al. (25) who have also shown that co-incubation of isolated mitochondrial with recipient cells results in the internalization of isolated mitochondria. The authors demonstrated that mitochondria isolated from renal proximal tubular cells were biologically active and capable of ATP production and that the isolated mitochondria could be actively internalized by renal proximal tubular cells in a dose dependent manner. The transplanted mitochondria increased proliferative capacity and ATP production and proliferation and significantly decreased cytotoxicity in an *in vitro* ischemia-reperfusion injury model.

Ali-Pour et al. (23) were also able to demonstrate mitochondria uptake into cardiomyocytes; however, in contrast to reports by others, increased bioenergetics lasted only 2 days (13, 19–21, 26, 27).

In our early experiments we used fluorescent mitochondrial specific labels such as MitoTracker CMXros and pHrodo to demonstrate mitochondrial uptake into cells (14, 20). The use of these fluorescent labels is informative but is not definitive as dissociation and re-association events have been postulated to occur.

To unequivocally demonstrate mitochondrial uptake, we have used human mitochondria for transplantation into animal models (13, 15, 26–30). The use of xenogeneic human mitochondrial transplantation in a rat, murine or swine model allows for the differentiation between native mitochondria and transplanted mitochondria based on immune reactivity to a monoclonal anti-human mitochondria antibody (24, 27). The use of human mitochondria in the rabbit and swine heart, kidney, lung and skeletal muscle has allowed us to track the fate of transplanted mitochondria across time. We use immunohistochemical selectivity to the human mitochondrial antibody as our primary marker with secondary markers of size and shape and function to confirm mitochondrial uptake. The transplanted human mitochondria in the rabbit and swine heart, kidney, lung and skeletal muscle induced no immune response as determined by ELISA and multiplex analysis and appear to maintain viability. Increased ATP content was detected at both 2 h and at 28 days after transplantation in the areas of mitochondrial transplantation (13, 15). No DAMPs (damage-associated molecular patterns) response or apoptosis or necrosis is evident in the areas receiving xenogeneic mitochondrial transplantation (13, 15, 26, 31).

It must be clearly noted that we do not recommend xenogeneic mitochondrial transplantation as the mtDNA differs and sufficient mitochondrial sources are available such that arguments for xenogeneic mitochondrial transplantation are moot (32).

2.1. Stability of functional integration

Our *in vivo* studies have shown that mitochondrial uptake is stable and can be visualized for at least 28 days post-transplantation in *in vivo* transplantation (13, 15, 26). The transplanted mitochondria are evident in both myocardial and non-myocardial cells at 2, 4, 8 and 24 h and at 28 days post-delivery. The exogenous mitochondria enhance cardiac function (increased left ventricular pressure, systolic shortening, decreased end diastolic pressure), enhance tissue viability (decreased tissue caspase 3 activity and necrosis) and enhance total tissue energy content (increased total tissue ATP content) following transplantation (13, 15, 26). These effects are evident at 2 h reperfusion and at 28 days recovery. Significantly our results show that there is no increase in peri-infarct size and that functional improvements in myocardial contraction remain intact throughout the recovery time of 28 days, verifying the enduring effects of mitochondrial transplantation on cellular viability and function.

2.2. Mechanism of uptake

The mechanisms for mitochondrial transplantation are distinct from mitochondrial transfer (20). Mitochondrial transfer involves the horizontal transfer of mitochondria from one cell to another. The transfer of mitochondria has been shown to occur through tunnelling nano tubes (TNT) which can occur either by uni- or bi-directional transfer (33). Spees et al. (34) demonstrated that co-culture of A549 cells with non-functional A549 p^o cells lacking mitochondrial DNA resulted in some of the non-functional cells acquiring functional mitochondria. This rescue was shown to occur through active mitochondrial transfer along cytoplasmic projections that made contact between donor and target cells. The authors were not able to establish whether mitochondria were transferred to the target cells directly through structures such as tunneling nanotubes or through uptake of vesicles containing mitochondria that budded off from the donor cells. Interestingly the authors showed that there was no passive transfer of mitochondria. Naked mitochondria isolated by differential centrifugation did not provide for rescue of A549 p^o cells.

Liu et al. (33) and Han et al. (35) were able to detect TNT-like structures allowing for intracellular transfer of mitochondria. Further studies by Berridge and Tan (36) and Tan et al. (37) confirmed the acquisition of mtDNA from host cells. Hayakawa et al. (22) have also shown that mtDNA and intact mitochondria can be transferred from other cells. These authors showed that extracellular mitochondria from astrocytes rescued neuronal viability and function.

3. Mitochondrial transplantation

3.1. Tissue source

The need for viable respiration competent mitochondria is essential for mitochondrial uptake and functional integration. In our initial publication on mitochondrial transplantation for cardioprotection we showed that the use of frozen mitochondria

with reduced mitochondrial oxygen consumption and membrane potential did not provide for ischemia- reperfusion protection (14). We also showed that the use of mitochondrial proteins, mitochondrial RNA and DNA or ATP did not provide for cardioprotection (14). These findings have been confirmed by Hayashida et al. (38) who have shown that frozen-thawed mitochondria have reduced membrane potential and were not efficacious for resuscitation following cardiac arrest in rats. This agrees with Kesner et al. (21) who have shown that disruption of the mitochondrial membrane decreases uptake of mitochondria into recipient cells. The importance of having intact respiration competent mitochondria has also been demonstrated by Cloer et al. (39) who have confirmed these earlier findings in a human DCD lung transplantation model where they showed that only intact mitochondria and not organelle secretions provided for therapeutic activity.

To allow for mitochondrial transplantation an appropriate tissue source must be available. Autologous tissue obtained from a non-ischemic site from the patient's own body offers the most clinically relevant source. In our studies, the source of tissue for mitochondrial isolation varies depending upon the incision site required for surgical access. This allows for therapeutic application without the need for secondary surgical intervention. In our procedures where a mini-thoracotomy or a sternotomy is performed, tissue from the pectoralis major or the rectus abdominus is obtained. When a carotid cut down is performed, tissue from the sternocleidomastoid can be obtained or when a femoral cut down is performed, tissue from the vastus medialis can be obtained. Other sources of tissue would depend on the incision site. The use of liver tissue may be appropriate when a laparotomy is performed.

A variety of cell lines have also been used as the source material for mitochondrial isolation. Pacak et al. (20) used mitochondria isolated from HeLa cells, Ali Pour et al. (23) used L6 skeletal cells, while Chang et al. (40) and Gollihue et al. (41) each used PC12 cells and Caicedo et al. (42) have used mitochondria isolated from mesenchymal stem cells. The potential for heterologous sources of tissue is great and could allow for readily available application in clinical settings and could allow for therapeutic treatment of mitochondrial associated mitochondrial myopathies.

We have used atrial appendage tissue, skeletal muscle, liver and cell culture as source material for mitochondrial isolation. We have found no advantage using organ specific or high or low glycolytic capacity mitochondria for ischemia-reperfusion protection (13–15, 20, 24, 26, 27).

Once exposed the tissue is dissected from the skeletal muscle using a number 6 biopsy punch. Usually two small pieces of tissue (>0.1 g) are harvested and stored in cold (4°C) phosphate buffered saline (clinical grade) and used for mitochondrial isolation. There are many methods for the isolation of mitochondria. The earliest published accounts of mitochondrial isolation date to the 1940s and these methods have been expanded upon and modified (43–45). Most mitochondria isolation protocols use tissue homogenization followed by differential centrifugation (46–48). Purification by Percoll gradient or sucrose step gradient centrifugation is often incorporated to purify the isolated mitochondria; however, the added centrifugation and washing time greatly extends the isolation process (49, 50).

3.2. Mitochondrial isolation

In our initial studies we isolated mitochondria using differential centrifugation (47, 48). The isolation of mitochondria was completed in approximately 90 min and required a starting tissue of approximately 5 grams (14). This amount of tissue is not available clinically and the viability of the isolated mitochondria using these methods is variable (51), for a more in-depth review of mitochondrial isolation techniques the reader is directed to (46–48, 52, 53). In addition, the time required for the isolation of mitochondria using this methodology would require extension of the surgical time and could result in complications that would be injurious to the patient (**Figure 1**).

To meet clinical demands, we have developed a novel methodology that allows for the rapid isolation and purification of mitochondria using differential filtration (53, 54). This methodology uses mechanical homogenization to minimize operator variability of homogenization. The tissue is obtained from the site of incision and is homogenized in a volume of 5 ml of sterile isolation buffer consisting of 300 mmol/L sucrose, 10 mmol/L HEPES-KOH [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid—potassium hydroxide], 1 mmol/L EGTA-KOH (Ethylenediaminetetraacetic acid—potassium hydroxide), pH 7.4, and then treated to 10 min of Subtilisin A enzymatic digestion, on ice. The digested tissue is then filtered through a series of filters by gravity filtration, and the mitochondria are subsequently precipitated by centrifugation at 9.5 × G for 5 min at 4°C (53, 54), https://sites.google.com/mccullylab.org/mccullylab). The time required for mitochondrial isolation using this procedure is 20–30 min and does not delay the surgical procedure.

The usual mitochondrial number obtained from the tissue samples (>0.1 g) using this methodology is $0.5–1.0 \times 10^{10}$ mitochondria. The isolated mitochondria are of the correct size and shape, as assessed by particle size counter and by transmission electron microscopy and have normal cristae and membranes and show no damage or injury (13, 24, 27, 28). The isolated mitochondria maintain membrane potential and oxygen consumption as determined by MitoTracker Red CMXRos staining and FACs analysis and mitochondrial complex I-V activity is maintained.

The isolated mitochondria have no detectable cytosolic, nuclear, or microsomal components that would include fragments from endoplasmic reticulum, endosomes, golgi apparatus, nucleus, and cytosol. We have performed enzymatic analysis of mitochondria isolates for detection of cytosolic and cytoplasmic contaminant markers glyceraldehyde 3-phosphate dehydrogenase and lactate dehydrogenase and the microsome contaminate markers 5´nucleotidase and glucose-6-phosphatase, and have found no detectable contamination by any of these contaminants. Western blot analysis also showed no detectable

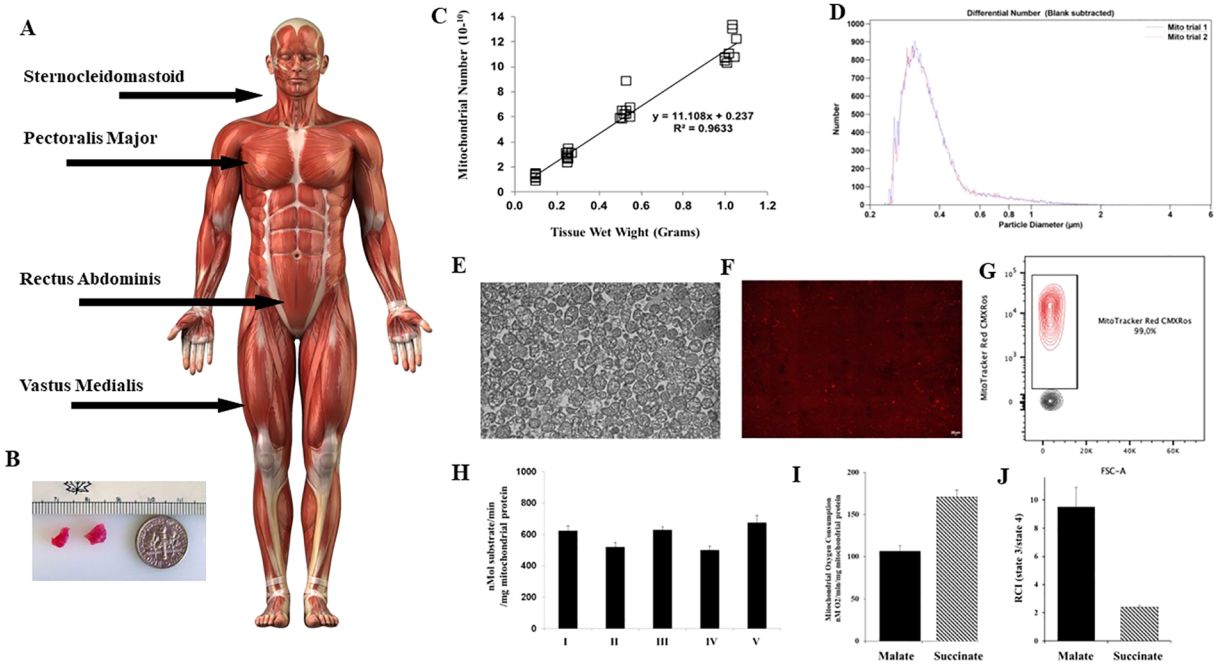

FIGURE 1
Mitochondria are isolated from tissue obtained at the site of the surgical incision. (A) Skeletal muscle tissue for mitochondrial isolation is shown at incision sites commonly used in cardiac surgery for sternotomy and mini thoracotomy. (B) Tissue is obtained using a #6 biopsy punch. Only two small pieces of viable tissue are needed. (C) From these two small pieces of tissue weighing approximately 0.1 gram 1 × 10 10 mitochondria can be obtained using the isolation methodology as described by (53, 54), and described at Available at: https://sites.google.com/mccullylab.org/mccullylab. (D) The isolated mitochondria are of consistent size as determined by particle counter. (E) The purity of the isolated mitochondria can be seen by transmission electron microscopy. (E) The mitochondria maintain membrane potential as determined by MitoTracker Red CmxRos staining. (F) Flow cytometry analysis of isolated mitochondria stained with Mitotracker Red CMXRos shows 98.6% of isolated mitochondria maintain membrane potential. (H) Complex I-V activity is maintained and (I) Mitochondrial oxygen consumption and (J) Respiratory Control Index (state 3/state 4) for malate induced (complex I) and succinate induced (complex II) is preserved.

presence of any contaminant markers in the isolated mitochondrial preparations (13, 24, 27, 28).

In all our studies, we have used total mitochondria for mitochondrial transplantation. The bioenergetic function of this population includes that of sub-sarcolemmal and intra-fibrillar mitochondria (55–57). We have found that total mitochondria offer the same level of cardioprotection as that provided by purified intrafibrillar or subsarcolemmal mitochondria sub-populations (14). The ease of obtaining total mitochondria and the reduced time for isolation make this choice clinically relevant.

3.3. Mitochondrial buffer for delivery

Following isolation, the mitochondria are suspended in buffer. In our early studies we suspended the isolated mitochondria in respiration buffer. This buffer contained 0.25 M sucrose, 0.002 M KH_2PO_4 (potassium dihydrogen phosphate), 0.1 M $MgCl_2$ (magnesium chloride), 0.2 M HEPES-KOH (pH 7.6), 0.0005 M EDTA-KOH (pH 8.0), 0.005 M glutamate, 0.005 M malate, and 0.001 M ADP (adenosine diphosphate) (14). Respiration buffer needs to be made fresh as the glutamate, malate and ADP can be degraded. Following several trials, we found that we could suspend the mitochondria safely in isolation buffer (300 mmol/L sucrose, 10 mmol/L HEPES-KOH, 1 mmol/L EGTA-KOH, pH 7.4) and that the isolated mitochondria maintained viability and function (15, 28, 58–60). The use of isolation buffer allows for ease of use in clinical applications as the multiple steps needed to make "fresh" respiration buffer are not required.

Modifications of this buffer have been used successfully by others. Hayashida et al. (38) have used buffer containing 250 mM sucrose, 2 mM KH_2PO_4, 10 mM $MgCl_2$, 20 mM K-HEPES-KOH (pH 7.2), 0.5 mM EGTA-KOH (pH 8.0). Huang et al. (61) have used 70 mM sucrose, 220 mM mannitol, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 2 mM HEPES, 1.0 mM EGTA and pH 7.2, for their studies in the rat. Gollihue et al. (41) suspend their isolated mitochondria in 215 mM mannitol, 75 mM sucrose, 0.1% bovine serum albumin, 20 mM HEPES, pH adjusted to 7.2 with KOH.

3.4. Stability after isolation

Our studies demonstrate that following isolation, oxygen consumption rate in isolated mitochondria is stable for at least 2 h with storage on ice. Mitochondrial state 3 oxygen consumption for malate (complex I) immediately following isolation was 106.6 ± 16.0 (nM O_2/min/mg mitochondrial protein). No significant difference in oxygen consumption rate was observed at 30, 60, 90, or 120 min after isolation with storage on ice. Oxygen consumption rate was 101.6 ± .17.3;

94.3 ± 12.3; 91.3 ± 15.6; and 94.6 ± 12.6 nM O_2/min/mg mitochondrial protein respectively. Oxygen consumption rate at 3 h storage was significantly decreased to 63.6 ± 24.6 nM O_2/min/mg mitochondrial protein. These results demonstrated that autologous mitochondria isolated using our methodology, in less than 30 min, were viable and suitable for clinical use for up to 2 h following isolation when stored on ice (**Figure 2**, unpublished data McCully et al.).

At present, only autologous tissue and cell culture have been used as sources for mitochondria isolation for use in mitochondrial transplantation. Cell culture as a source for mitochondrial isolation could provide a ready and available source of mitochondria that could be used for immediate use in settings such as acute heart attack. The best scenario would be to have an "of-the-shelf" product that would allow for immediate usage, however, the methodology for such a product remains to be developed.

3.5. Delivery methods

A variety of delivery methods for mitochondrial transplantation have been developed and these range from simple naked co-incubation to sophisticated mechanical delivery. Chang et al. (40) have proposed the use of a cell-penetrating peptide (Pep-1) to aid in the uptake and internalization of mitochondria. The authors used Pep-1 conjugated mitochondria in a rat model of Parkinson's disease and have reported improvement of rotational and locomotor behaviors in this model.

Liu et al. (62) have developed a method where isolated mitochondria are linked to a carrier that allows the systemically injected mitochondria to be directed to the liver. The authors used asialoglycoprotein linked to listeriolysin O to form complexes with freshly isolated liver mitochondria. This complex forms a mitochondrial-carrier protein capable of being recognized, bound, and internalized by asialoglycoprotein receptors in the liver. Listeriolysin O was added to facilitate the release of internalized mitochondria from endosomes. Using this complex the authors reported targeted delivery of mitochondria by intravenous delivery in the rat with 27% of the injected mitochondria being found in the liver.

Kim et al. (63) have shown in cell cultures that isolated mitochondria can be transferred into recipient cells by simple low speed [1,500 × g (gravity) for 5 min at 4°C.] centrifugation. The transferred mitochondria maintained bioenergetic function and increased intracellular ATP content and metabolic activity, and also delivered mtDNA to the recipient cells.

MitoCeption, proposed by Caicedo et al. (42) also uses centrifugation but adds thermic shock to enhance mitochondria uptake in cells. The authors added isolated mitochondria to cells grown on a cell plate surface. The culture plates were then centrifuged at 1,500 × g for 15 min at 4°C. They were then placed in a 37°C cell incubator for two hours, prior to a second centrifugation. The authors showed that MitoCeption increased mitochondrial DNA concentrations, oxygen consumption rate and ATP production.

Mechanical approaches have also been proposed to facilitate mitochondrial delivery. Wu et al. (64) have proposed the use of a photo-thermal nanoblade which induces a transient membrane opening to allow for mitochondrial uptake into cells. These transient and localized openings are thought to allow for specific uptake of mitochondria. In another modification for mitochondrial uptake Wu et al. (65) have proposed the use of a

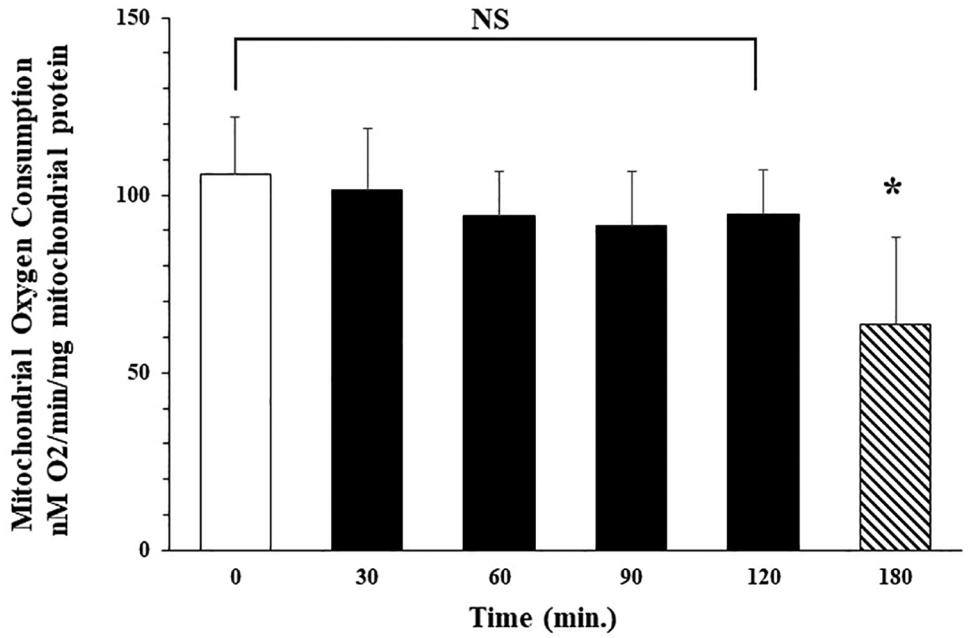

FIGURE 2
Isolated mitochondria are stable at 4°C for 120 min No significant difference (NS) was observed in mitochondrial oxygen consumption at 0, 30, 60, 90 or 120 min after isolation. Mitochondrial oxygen consumption significantly decreases at 180 min * = $p < 0.05$ vs. time 0.

stamp-type multi-needle injector. The device has been used for the delivery of mitochondria to restore aging-related hair loss.

Macheiner et al. (66) have developed Magnetomito Transfer which uses a mitochondrial specific antibody to TOM-22 linked to an iron bead that binds to the outer mitochondrial membrane to stimulate mitochondrial uptake under a magnetic field. The methodology was developed to allow for transfer of healthy allogenic mitochondria to a patient's own stem cells prior to autologous stem cell transplantation. The authors report a trend for higher density of mitochondria in magnetomito-transferred cells.

Recently, Yang et al. (67) have suggested the use of gelatin nanospheres to enhance mitochondrial uptake into cells. In their studies isolated mitochondria were first modified by electrostatic attachment of gelatin nanospheres to the outside of the mitochondria. The nano-sphere-mitochondria were then co-incubated with H9c2 cardiomyoblasts where functional uptake was confirmed by ATP synthesis.

Another approach to delivery of genes or pharmaceuticals to the mitochondria is Mito-porter developed by Yasuzaki et al. (68). This methodology does not use mitochondria but rather uses a liposome-based carrier that permits macromolecular cargos to enter mitochondria via membrane fusion. The authors have used mito-porter in a series of studies to demonstrate that mtDNA and other products can be delivered effectively to the mitochondria (69).

3.5.1. Co-incubation

For cell culture studies, most authors resuspend the isolated mitochondria in media. In our studies the cell culture media Dulbecco's Modified Eagle's Medium contains 1.8 mM calcium, We and others (13, 20, 21, 70) have found no stability problems of the isolated mitochondria in these physiological calcium concentrations.

3.6. Stability in Serum and blood

Shi et al. (71) investigated isolated mitochondrial stability in rat serum. The authors added isolated mitochondria to mouse blood serum and incubation at the mixture at 37°C for 0, 15, 30, 60, 120 min, respectively. The authors then measured mitochondrial viability using MitoTracker Red CMXRos and mitochondrial membrane potential at each time point. The authors reported that there was no difference in mitochondrial viability or mitochondria membrane potential ($\Delta\Psi m$) observed at the different time points (0 min, 15 min, 30, 60 and 120 min) suggesting that the mitochondria remained stable in serum for at least 120 min.

Intact mitochondria have been detected in plasma in healthy patients and in patients having pathological conditions. Proof for the existence of cell-free respiratory competent mitochondria in blood was first reported by Amir Dasche et al. (72). The authors showed mitochondria from a normal cell line (CCD-18Co) and from human colon cancer cell lines (DLD-1/SW620) were able to secrete their mitochondria. The authors went on to show the presence of circulating cell-free mitochondria in healthy individuals and in cancer patients. The cell free circulating mitochondria were structurally intact and not fragmented and had preserved mtDNA integrity and were not surrounded by bi- or multi-layer phospholipid membrane, supporting the observation that they were free circulating mitochondria and not enclosed in extracellular vesicles such as exosomes or microvesicles or autophagosomes. The cell free circulating mitochondria were also respiration competent.

These findings have been replicated by Stephens et al. (73) who showed that intact, cell free mitochondria are present in blood and these mitochondria maintained transmembrane potential and were able to reenter cells.

Amir Dasche et al. (72) and Stephens et al. (73) estimated the presence of cell-free and respiratory competent mitochondria in human blood to be between 200,000 to 3.7 million per mL and 822,000 to 2.3 million per mL plasma in humans, respectively. The mechanisms through which mitochondria are able to survive in serum and their relevance in non-pathological human organ maintenance are unknown.

3.7. Direct injection

Direct injection into the tissue is the simplest methodology for clinical delivery of mitochondria for transplantation. For direct injection, the isolated mitochondria are suspended in buffer and are delivered to the organ using a 1 ml tuberculin syringe with a 28 or 32 gage needle. The mitochondria can be delivered directly to affected areas in the organ. In the heart this may be areas of hypo-kinesis or a-kinesis as determined by epicardial echocardiography. The mitochondria are delivered in 50–100 ul injections. This volume is rapidly taken up by the neonatal, pediatric and adult myocardium and no flushback occurs and there is no need for purse string sutures. Similar uptake is observed in skeletal muscle. The angle of injection is not limited to oblique delivery as in stem cell injection (13–15, 26, 27, 29, 30, 74, 75).

The isolated mitochondrial dosage amounts in the heart were determined to be 2×10^5 to 2×10^6 mitochondria per gram tissue wet weight (13, 14, 28). Mitochondrial concentrations $>2 \times 10^8$ were not consistently fully suspended in vehicle buffer.

Direct injection of mitochondria has no effect on arrhythmogenicity (13). Our studies in the *in vivo* heart have demonstrated that there is no proarrhythmia as determined by serial ECG, no change in QRS duration and no change in corrected QT interval. Serial electrocardiography and echocardiography also demonstrated that there was no evidence of wall motion disturbances, no evidence of left ventricle hypertrophy, valve dysfunction, fibrosis or pericardial effusion at 4 weeks following transplantation of mitochondria.

To ensure no proarrhythmia was associated with mitochondrial transplantation we also performed optical mapping studies using isolated mitochondria at a concentration of 8.4×10^7/gram tissue wet weight as compared to 2×10^5 to 2×10^6/gram heart wet weight. This concentration of mitochondria was used in order to

detect any possible arrhythmogenic response (13). Our results demonstrated that there was no change in isopotential mapping associated with mitochondrial transplantation and confirming that mitochondrial transplantation is not proarrhythmic.

3.8. Intra-arterial delivery

Intra-arterial delivery of mitochondria simplifies mitochondrial delivery to tissue and allows for widespread distribution of mitochondria within the tissue. Intra-artery delivery to the heart is achieved via carotid cutdown (28). For the lung we deliver via the pulmonary artery (76) while for the kidney we perform a femoral artery cutdown (29).

The artery is exposed and accessed, and an arterial sheath is positioned in the artery and an angiography catheter is introduced through the arterial sheath, and floated to the delivery site, the coronary ostium in the case of the heart or the infrarenal arteries in the case of the kidney. This procedure is performed under fluoroscopy (28, 29, 58, 77). A 4 s or 5s catheter can be used. The isolated mitochondria are suspended in 5 ml of buffer and injected as a bolus and then chased with 5 ml of buffer.

To ensure that intra-coronary delivery was safe and did not affect coronary patency we have performed *in vivo* studies in the pig heart (28). The pig heart provides a standardized model for the human heart having similar vascular architecture, capillary diameter and morphology as that in the human heart (78, 79). In these studies, intra-coronary delivery was investigated using mitochondria concentration of 1×10^3 to 1×10^{11} in the presence of increased myocardial demand, coronary vasoconstriction or tachycardia with increased afterload. Our results demonstrated that intracoronary injection of mitochondria at concentrations of 1×10^3 to 1×10^{11} has no adverse effects on coronary patency, cardiac rhythm, or function. We also showed that mitochondria can be safely injected into severely constricted coronaries as well as under significant hemodynamical stress of tachycardia and hypertension, all of which often accompany various pathological conditions of the heart. As with direct injection of mitochondria there is no proarrhythmia associated with intra-arterial delivery of mitochondria.

3.9. Aerosol delivery

To increase applicability and allow for early, non-surgical intervention and post-surgical intervention we have extended our research to show that autologous mitochondria can be delivered by nebulizer to the lungs. In a study of acute lung injury, we compared aerosol delivery of naked mitochondria via the trachea and intra-vascular delivery, via the pulmonary artery (76). Vascular delivery of mitochondria to the left lung was achieved by injection of mitochondria in buffer directly into the left pulmonary artery. Aerosol delivery of mitochondria was achieved by nebulization using the FlexiVent nebulizer system (FlexiVent FX2, SCIREQ, Montreal, Quebec, Canada). Our results demonstrated that delivery of mitochondria by pulmonary artery infusion and by nebulization was efficacious.

3.10. Nasal delivery

In the brain, delivery of exogenous mitochondria to the ventricles has been shown to be effective, but clinically, the impermeability of the blood-brain barrier poses a major restriction for use. Orreogo et al. (80) have reported that the delivery of mitochondria to the brain can be achieved using osmotic disruption of the blood-brain barrier followed by carotid artery infusion of the mitochondria. The authors report that in the absence of osmotic disruption, few mitochondria were detected in the brain but following osmotic disruption, transplanted mitochondria were detected in all regions of the cortex and across the parenchyma.

Alexander et al. (81), and Alexander et al. (82), have shown that nasal administration of mitochondria isolated from human mesenchymal stem cells restored executive functioning, working and spatial memory in mice with cisplatin-induced cognitive deficits. The authors showed human mitochondria gained rapid entry into the brain of mice. The transplanted mitochondria were seen entering the brain from the pia mater v and glial limitans and were present at 30 min and 18 h after administration and repaired cisplatin-induced loss of white matter integrity and synaptic damage. The transplanted mitochondria also induced transcriptomic alterations in the hippocampus, up-regulating cognitive related restorative pathways. These findings agree with Chiu et al. (83) and Galeano et al. (84) and Danielyan et al. (85) who have each shown uptake of nasal delivered mesenchymal stem cells in the brain.

3.11. Delivery to the spinal cord

Delivery of mitochondria to the spinal cord has been shown to be troublesome. Golihue et al. (41) studied mitochondrial transplantation in a rat model of spinal cord injury. The authors showed that exogenous mitochondrial were taken up by the injured spinal cord and were evident at 24- and 48 h, and 7 days post-injection and increased bio-energetics. The mitochondria co-localized with multiple resident cell types, although they were absent in neurons. Unfortunately, mitochondrial transplantation did not yield long-term functional neuroprotection as assessed by overall tissue sparing or recovery of motor and sensory functions. This group have since gone on to promote new techniques for mitochondrial transplantation for spinal cord injury (86).

4. Uptake occurs in healthy cells and in ischemic cells

The uptake of mitochondria has been shown in many different cell types and pathologies. In our studies we have found that mitochondria are taken up by all cell types. To visualize

mitochondrial uptake and distribution subsequent to direct injection or infusion through the coronary vasculature, we have labelled mitochondria with ^{18}F-Rhodamine-6G (^{18}F-R6G) or ^{18}F-R6G and iron oxide nanoparticles (27–30, 87). The distribution and uptake of labelled mitochondria was determined by positron emission tomography (PET), microcomputed tomography (uCT), and magnetic resonance imaging (MRI) with subsequent microscopic analyses of stained tissue sections to confirm the uptake and distribution of transplanted mitochondria (27). In these studies, we showed that direct injection of mitochondria resulted in discrete localized uptake of the labelled mitochondria while vascular delivery of mitochondria through the coronary arteries resulted in their rapid integration and widespread distribution throughout the heart. Both modes of delivery provided cardioprotection from ischemia-reperfusion injury by significantly preserving systolic shortening and cell viability.

Notably we showed that mitochondria are taken up in myocardial areas that were not subjected to regional ischemia and reperfusion. The uptake in non-ischemic areas was less than that observed in the regional ischemic area in the heart, and we speculate that this increased organelle uptake was due to myocardial cell swelling and loss of cellular integrity.

5. Dosage

The dosage of mitochondria varies in different studies. Both mitochondrial protein and absolute mitochondrial number have been used to standardize dosage (13, 19, 21, 23, 41). In all our studies we have used particle counting to estimate the dose of mitochondria used. Our studies have shown that mitochondria concentrations of 2×10^5 to 2×10^6 mitochondria per gram wet weight heart tissue is efficacious (13, 26, 28, 58, 77). This represents approximately 1×10^9 mitochondria in the adult 400 g heart. Concentrations less than 2×10^5 to 2×10^6 mitochondria per gram wet weight heart tissue were associated with decreased cardioprotective efficacy while concentrations greater than 2×106 per gram wet weight heart tissue failed to increase efficacy (28). We have found that this dosage concentration is also applicable to the kidney (29) and the lung (76); however, for skeletal muscle increased mitochondrial concentrations were required (30).

Our studies have shown that only a small number of mitochondria are needed to alter organ function. These studies and those of others suggest that the number of mitochondria required for cardioprotection is not a function of the absolute number of mitochondria residing within the cell (14, 28, 88, 89). This would agree with Shoffner et al. (90) who have demonstrated that only 2%–6% of mtDNA needs be wild type to alter oxygen uptake and the devastating effects of the mitochondrial myopathy MERRF (Myoclonic epilepsy and ragged-red fiber disease) and with Chomyn et al. (91) who have reported that levels of only 6% wild type mtDNA are sufficient to modulate the effects of MELAS (Mitochondrial Encephalopathy, Lactic acidosis, and Stroke-like episodes).

6. Safety of mitochondrial transplantation

Enzyme-linked immunosorbent spot (ELISpot), enzyme-linked immunosorbent assay (ELISA), fluorescence-activated cell sorting (FACS) and multiplex analysis has demonstrated there is no direct or indirect immune response and there are no inflammatory effects associated with mitochondrial transplantation (13, 26, 28, 31).

Masuzawa et al. (13), showed using serial ELISA analysis that there was no significant increase in TNFα, IL-6 or high-sensitive c-reactive protein at 1-, 3- and 7- days post mitochondrial transplantation and provided evidence suggesting that the level of inflammation was ameliorated by mitochondrial transplantation. RNAseq analysis confirms this observation as a cardio-protective mechanism associated with mitochondrial transplantation in the heart (15).

To confirm the ELISA results Masuzawa et al. (13) also performed multiplex analysis of cytokines and chemokines. In this assay, both intact mitochondria and sonicated mitochondria were used with *in vitro* analysis. The sonicated mitochondria were used to determine the effects of mitochondria degradation products. Separate analysis was performed to determine innate chemokine and cytokine activation in human peripheral blood mononuclear cells. Masuzawa et al. (13) showed that there was no upregulation of cytokines associated with the immune response (IL-1, IL-4, IL-6, IL-12, IL-18, IP-10, macrophage inflammatory protein (MIP-1α and MIP-1β). Importantly, Masuzawa et al. (13) showed that mitochondrial transplantation upregulated epidermal growth factor (EGF), growth-related oncogene (GRO), IL-6 and monocyte chemotactic protein-3 (MCP-3). These cytokines have been shown to be associated with enhanced post-infarct cardiac function.

Ramirez-Barbieri et al. (31) investigated alloresponse and allorejection to syngeneic and allogeneic mitochondrial transplantation. This study examined immune response to single injections of mitochondria at concentrations of 1×10^5, 1×10^6 and 1×10^7 and serial injections given a concentration of 1×10^7 mitochondria on days −6, −3 and day 0. These concentrations are equivalent to 10-, 20- and 30-fold, and 90-fold respectively, the concentration of mitochondria used in our animal and clinical studies (13, 14, 26–28, 58, 74, 77, 92). Ramirez-Barbieri et al. (31) investigated both syngeneic and allogeneic mitochondria. Experiments were conducted using the BALB/cJ mouse strain to allow for human relevance. Allogeneic mitochondria were obtained from C57BL/6J mice. Mitochondria were delivered by intraperitoneal injection to maximize immune reaction. This was done as previous studies have shown that by intraperitoneal injection elicits a greater immune reaction than either intra-venous or direct injection by creating a greater distribution of the antigen to the lymph nodes and to different organs in the body. Immune response was measured at 10–17 days post-injection as the immune response in the BALB/cJ mouse strain is not evident prior to 7 days post antigen presentation.

Using this stringent protocol, Ramirez-Barbieri et al. (31) were able to show that there was no detectable direct or indirect B-cell or T-cell response as determined by ELISpot and FACS analysis and that multiplex analysis did not detect any increase in any of the cytokines or chemokines associated with the innate or acquired immune response for either syngeneic or allogeneic mitochondrial transplantation. Ramirez-Barbieri et al. (31) also showed there was no mitochondrial DAMPs (damage-associated molecular patterns) response associated with mitochondrial transplantation, no evidence of myocardial cellular damage or increased collagen content and no increase in circulating free mitochondrial DNA.

Masuzawa et al. (13) also showed there was no autoimmune response to mitochondrial transplantation and that there were no detectable anti-mitochondrial antibodies.

7. Biodistribution

Direct injection, intra-coronary, pulmonary and intra-renal artery infusion of mitochondria have been shown to provide discrete mitochondrial uptake. Once delivered the mitochondria rapidly enter the cells and remain present for at least 28 days, the terminal time for our animal experimentation. In a series of studies, separate analysis using autologous or xenogeneic human mitochondria each labelled with ^{18}F-Rhodamine-6G (^{18}F-R6G) have been performed. Results from these studies, in the *in vivo* swine model, have shown that the mitochondria are rapidly taken up by the cells in the end organ and are not present in other tissues. This localization of mitochondria following delivery was confirmed using mitochondria at concentrations 6-fold greater than we recommend. In our heart studies PET imaging demonstrated that intracoronary delivery distributes mitochondria specifically to the cardiac vascular supply, displaying signals only in the left ventricle when mitochondria were injected into the left coronary ostium. The tracer signal was not detected in other organs, despite the injection of much higher concentrations (six fold) of mitochondria than that used for therapeutic dosage. Similar findings were observed in the lung by pulmonary artery delivery (76), and in the kidney by intra-renal infusion (29), respectively.

Our studies show that in the heart, lung and kidney mitochondria rapidly crossed the vascular endothelial cells. ^{18}F-Rhodamine-6G labeled mitochondria were found inside cardiomyocytes at 10 min following coronary artery delivery, while in the kidney the mitochondria were distributed throughout the tubular epithelium of cortex and medulla following renal artery delivery (28, 29). In the lung the transplanted mitochondria were detected within and around lung alveoli and connective tissue (76).

The mechanism(s) of vascular extravasation of mitochondria remain to be fully elucidated; however, the rapidity of mitochondria transport to cardiac cells is likely to involve mechanisms similar to bacterial or viral uptake.

8. Mechanisms of mitochondrial transplantation

We now have sufficient experimental proof to speculate on the overall mechanisms of mitochondrial transplantation.

8.1. Reactive oxygen Species

McCully et al. (14) investigated some of the mechanisms that may be involved in mitochondrial transplantation. These early experiments showed there was no increase in organ specific reactive oxygen species associated with mitochondrial transplantation. Thiobarbituric acid reactive substances (TBARS) assay, of lipid peroxidation showed that mitochondrial transplantation significantly decreased reactive oxygen species. Collaborative experiments using the reactive oxygen species scavenger, *N*-(2 mecaptopropionyl) glycine (MPG) when used throughout reperfusion or when added to mitochondria also failed to block the cardioprotection afforded by mitochondrial transplantation. Myocardial cell function as determined by sonomicrometry and cellular viability (necrosis and apoptosis) by triphenyl tetrazolium chloride staining was not altered with the addition of MPG.

These studies showed that mitochondrial transplantation did not increase reactive oxygen species (ROS) and that ROS was not involved in the mechanisms associated with mitochondrial transplantation. These initial findings agree with that of Kim et al. (63) who showed that mitochondrial delivery via centrifugal force did not cause intracellular damage, increase in oxidative stress (intracellular ROS and mROS) or apoptosis. Recently, Rossi et al. (25) have also shown that mitochondrial transplantation was associated with lower ROS production. The authors showed that mitochondrial transplantation decreased ROS generation as determined by the ROS-sensitive fluorescent probe MitoSOX together with the coherent decrease of TBARS production. In total these studies indicate that the effects associated with mitochondrial transplantation are not modified by reactive oxygen species. We have done no experiments using anti-oxidant enzymes.

8.2. Inflammation

It has been suggested that inflammation due to an acute immune response and inflammatory macrophage activation may play a role in tissue repair (93). Masuzawa et al. (13) has shown using serial blood samples over 4 weeks of recovery, that TNFα, IL-6 and high sensitivity C-reactive protein, sensitive markers of inflammation were significantly decreased in hearts receiving mitochondrial transplantation as compared hearts receiving vehicle alone suggesting that the level of inflammation was ameliorated by mitochondrial transplantation. Similar results were observed by Kaza et al. (26) who, using multiplex assay showed that there was no

immune or inflammatory response or cytokine activation associated with mitochondrial transplantation.

Ramirez-Barbieri et al. (31) have shown by ELISpot assay that inflammatory cytokines INFγ, IL-2 (type 1 cytokines) were not increased with mitochondrial transplantation even at 90- fold the recommended mitochondrial concentration. Ramirez-Barbieri et al. (31) also examined cytokine profiles, involving a population of macrophages, Th1, Th2 cytokines. Multiplex analysis demonstrated that there was no detectable increase in the levels of any cytokine for either syngeneic or allogeneic mitochondria transplantation at any mitochondrial concentration (1×10^5, 1×10^6 or 1×10^7). Guariento et al. (94) have shown in a clinical study of patients requiring extracellular membrane oxygen support for postcardiotomy ischemia-reperfusion injury, that mitochondrial transplantation was not associated with inflammatory response. In total, these studies indicate that the effects associated with mitochondrial transplantation are not modified by inflammation.

8.3. Adenosine receptors and K_{ATP} channels

An interesting phenomenon was observed with intracoronary delivery of mitochondria, namely a sustained increase in coronary blood flow. This effect on coronary blood flow was immediate and concentration-dependent, with maximal hyperemia achieved with an intracoronary injection of 1×10^9 mitochondria (28). The increase in coronary blood flow was also accompanied by an increase in systolic shortening with no change in heart rate or mean arterial pressure. The mitochondria-induced increase in coronary blood flow was achievable only through intracoronary delivery of intact, respiration-competent mitochondria. Direct injection of mitochondria into the heart muscle or intracoronary delivery of devitalized mitochondria or intact HeLa p0-mitochondria, which lack respiration capacity, had no effect on coronary blood flow or systolic shortening. This finding is consistent with earlier findings by us and others that the transplanted mitochondria must be intact and respiratory competent.

The mechanism for this was found not to be attributable to ATP (adenosine triphosphate) produced by the mitochondria or changes in oxygen saturation. In vivo inhibition of key coronary vasodilatory pathways: nitric oxide synthase (NOS), cyclo-oxygenase (COX), adenosine-receptors, potassium-ATP (K_{ATP}) channels and oxygen saturation were also found to have no effect on the changes in coronary blood flow and systolic shortening. Interestingly, mitochondria-induced coronary vasodilation was attenuated in part by the inhibition of the inward-rectifying potassium (K_{IR}) channels, consistent with studies that implicate K_{IR}-channels in mechanisms of ATP-mediated vasodilation (28). These results are in agreement with earlier studies (14) that showed that in the Langendorff perfused heart with the non-selective adenosine receptor inhibitor 8-sulfophenyltheophylline or the non-selective potassium-ATP (K_{ATP}) channel blocker glibenclamide or pre-incubation of isolated mitochondria with these drugs had no effect on the observable increases in systolic shortening and decreased infarct size obtained by mitochondrial transplantation (**Figure 3**). For review of the role of potassium-ATP (K_{ATP}) channels the reader is directed to (8, 95).

8.4. Chemokines, cytokines

Masuzawa et al. (13) showed that there was up-regulation of cardioprotective cytokines. These cytokines, epidermal growth factor (EGF), GRO, IL-6 and monocyte chemoattractant protein-3 (MCP-3) have been shown to play key roles in angiogenesis, arteriogenesis, immunomodulation, progenitor cell migration, prevention of apoptosis and enhanced cell salvage and post-ischemic functional recovery. EGF has been shown to play a key role in ischemic injury protection in the heart by stimulating cell growth, proliferation, and migration. After cardiac infarction, GRO participates in the improvement in function and reconstitution of tissue mass and acts with IL-6 as a chemo-attractant which allows for enhanced vascularization, protection against cardiomyocyte apoptosis, and improved functional cardiac recovery. These chemokines have been shown to act with MCP-3 to enhance post-infarction cardiac function and improve cardiac remodeling independent of cardiac myocyte regeneration.

8.5. ATP

We and others have shown that mitochondrial transplantation improves bioenergetics and oxygen consumption both *in vitro* and *in vivo* (19–21, 23, 25). These beneficial effects are dependent upon the respiratory capacity and integrity of the transplanted mitochondria (13, 21, 28, 39).

We have demonstrated in the *in vivo* model that the mechanism of action of mitochondrial transplantation involves in part the prolonged increase in total tissue ATP content. Doulamis et al. (15) has shown that in the *in vivo* heart, mitochondrial uptake in the area at risk is evident at 2 h, 3 days and at 28 days post mitochondrial transplantation and is associated with significantly increased total tissue ATP content at both 2 h and at 28 days. The mechanism for this increase has been investigated by Rossi et al. (25) who have shown that mitochondria transplantation in a model of ischemia-reperfusion injury was able to restore the activity of the TCA cycle enzymes citrate synthase, alpha-ketoglutarate, succinate, and malate dehydrogenase and the enzymes of the electron transport chain, leading to increased intracellular ATP levels such that there was no difference as compared to non-ischemic controls. This agrees with the findings of Masuzawa et al (13), who showed that oxygen consumption rate in cardiomyocytes was significantly increased with mitochondrial transplantation and with Guariento et al. (58) who showed that mitochondrial transplantation significantly increased myocardial oxygen consumption.

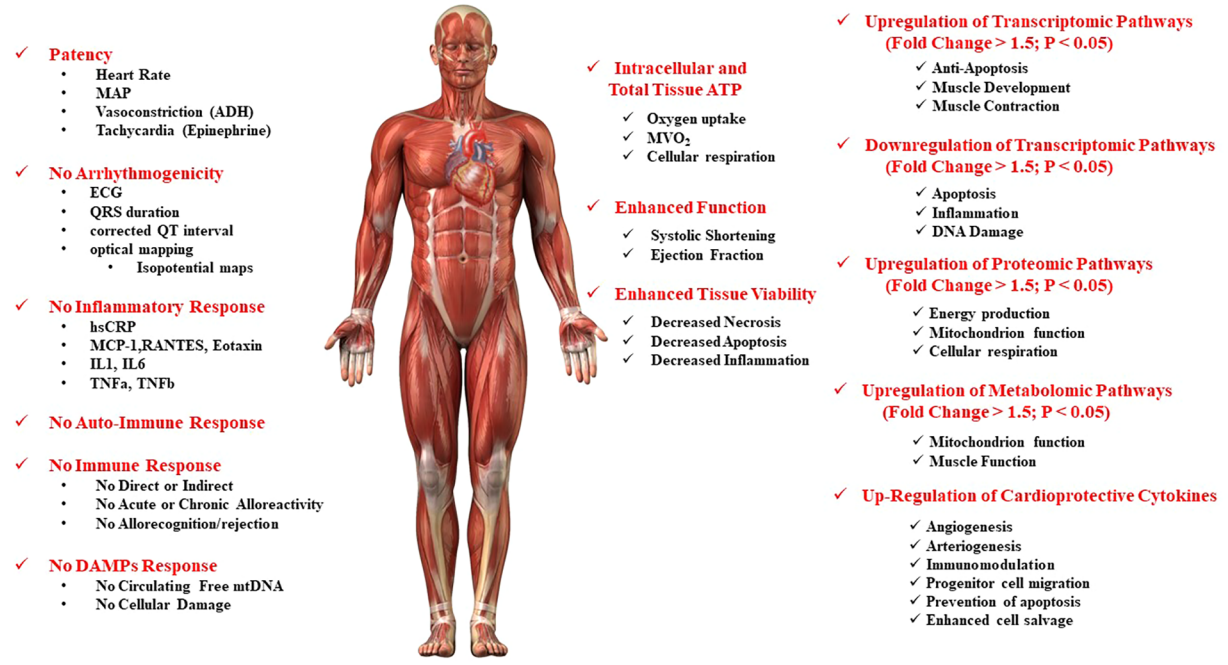

FIGURE 3
Safety and putative mechanisms associated with mitochondrial transplantation in the ischemia-reperfusion injured heart.

8.6. Transcriptomic, proteomic and metabolomic responses to mitochondrial transplantation

Previously we have shown by microarray and proteomic analysis that cardioprotection following ischemia reperfusion injury is modulated by RNA- and protein-dependent mechanisms (12, 96). Transcriptomic and proteomic enrichment analyses indicated that ischemia downregulated genes/proteins associated with mitochondrial function and energy production, cofactor catabolism, and the generation of precursor metabolites of energy. In contrast, cardioprotection with cardioplegia significantly increased differentially expressed genes/proteins associated with the mitochondrion and mitochondrial function and significantly upregulated the biological processes of muscle contraction, involuntary muscle contraction, carboxylic acid and fatty acid catabolic processes, fatty acid b-oxidation, and fatty acid metabolic processes (12, 96). The transcriptomic and proteomic data demonstrated that the mitochondrion plays a significant role in both ischemia and in cardioprotection.

To ascertain the early expressed underlying global transcriptomic, proteomic and metabolomic changes conferred by mitochondrial transplantation we have performed RNAseq, SOMAscan and Metabolomic analysis to identify pathways up- and down-regulated with mitochondrial transplantation.

Masuzawa, et al. (13) showed that mitochondrial transplantation was associated with up-regulation of proteomic pathways. These *in situ* experiments demonstrated that mitochondrial transplantation beneficially altered proteomic pathways early in reperfusion, allowing for enhanced post-ischemic functional recovery and enhanced post-ischemic myocellular viability. Functional annotation clustering ($p < 0.05$, Enrichment Score > 2.0) indicated that the mitochondrion, the generation of precursor metabolites for energy and cellular respiration were enriched with mitochondrial transplantation and there were no down regulated clusters.

Doulamis et al. (15) also showed that mitochondrial transplantation up-regulated (fold change >1.5, $p < 0.05$) proteomic pathways for multicellular organismal processes, response to organic substance, stimulus and external stimulus, and multicellular organ and system development at 2 h and at 28 days recovery. All these pathways are associated with mitochondrial function and biosynthesis. Gene ontology localization analysis showed that the modulations in transcriptomics were affected by mitochondrion. No other organelle was implicated.

Doulamis et al. (15) showed that the changes in transcriptomics were consistent with proteomic alterations and showed that biological processes for regulation of multicellular organismal processes, regulation of biological quality, regulation of system processes, regulation of signaling, response to organic substance and response to oxygen containing compound were significantly upregulated in both RNA-seq and proteomic analysis.

These studies agree with the earlier studies by McCully et al. (96) and Black et al. (12) confirming the role of the mitochondrion in ischemia and in cardioprotection. These studies are also consistent with the findings of Rossi et al. (25) who have shown that mitochondrial transplantation up-regulates transcriptomic pathways associated with mitochondrial biogenesis [Peroxisome proliferator-activated receptors (PPAR) pathway],

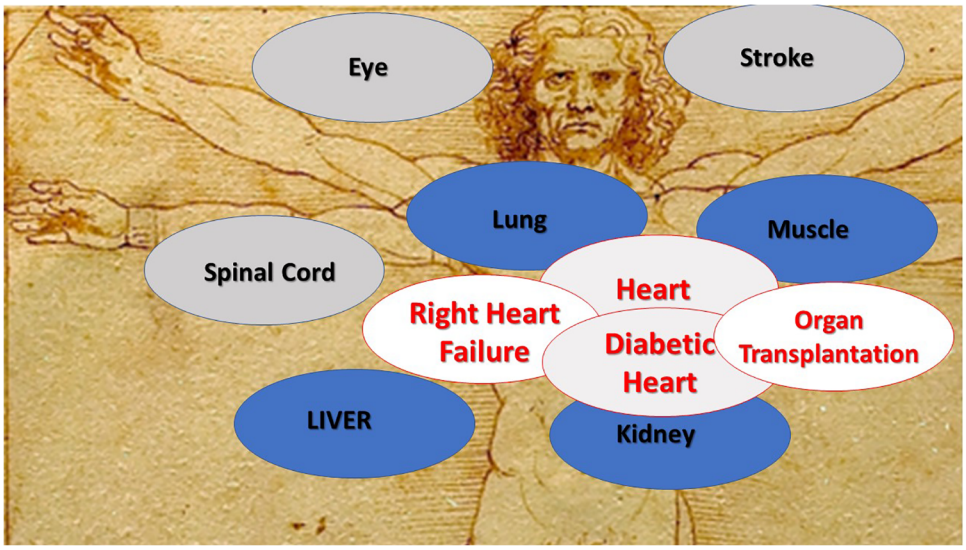

FIGURE 4
Clinical uses for mitochondrial transplantation.

mitochondrial metabolism (IL-17, Ca2+, cAMP, and cAMP response element (CREB) signaling.

Cumulatively, these findings support the observations of Guariento et al. (58) who demonstrated that mitochondrial transplantation enriched metabolomic pathways for mitochondrion function and muscle function.

The physiological, functional, and biochemical results obtained in our animal studies in the ischemic-reperfused heart model support the pathways suggested by transcriptomic, proteomic and metabolic analysis. Our data clearly show that there is no immune or inflammatory response associated with mitochondrial transplantation. In agreement with the findings of Alexander et al. (81) who investigated mitochondrial transplantation by nasal delivery and showed that there was no evidence for activation of inflammatory pathways in the brain following mitochondrial transplantation and that there was no up-regulation of transcriptomic inflammatory signaling. The authors showed that the top canonical pathways upregulated by the nasal administration of mitochondria were the Nrf2-mediated oxidative stress response, along with telomerase, ERK/MAPK and synaptogenesis signaling. It was speculated that Nrf2-mediated response may regulate antioxidant proteins towards minimizing oxidative damage and that protein repair and clearance may also be triggered by ubiquitination, proteosome degradation and regulation of chaperone and stress response proteins. The authors suggested that mitochondrial transplantation may repair the acceptor cells like neurons, macrophages, and GFAP + cells possibly by changing their metabolic programming towards restoration of the damage and/or a more restorative phenotype.

The down-regulation of pathways for DNA damage are supported by the findings of Pacak et al. (20) who showed that mitochondrial transplantation rescued cell function and replaced mtDNA.

Down-regulation of pathways for proteolysis and apoptosis and up-regulation of the pathway for anti-apoptosis with mitochondrial transplantation also agree with our findings that mitochondrial transplantation significantly decreases myocardial necrosis and apoptosis (13–15, 26–28, 58, 77). Reduction in myocardial injury has been confirmed by significant decreases in CK-MB and cTnI and a significant decrease in caspase-3 like activity.

Up-regulation of transcriptomic pathways for muscle contraction and muscle development and proteomic pathways for muscle function and metabolomic pathways for muscle function also agree with our measured contractile indices where we have shown that mitochondrial transplantation enhances post-ischemic myocardial contractile function that includes increased left ventricular developed pressure, maintenance of left ventricular end diastolic pressure, increased systolic shortening, increased ejection fraction.

The up-regulation of proteomic and metabolomic pathways for energy production, mitochondrial function, cellular respiration and mitochondrial function and the metabolic pathway for mitochondrial function agree with our studies showing that mitochondrial transplantation increases total tissue ATP content, MVO2 and cellular respiration.

In total, these data show that the responses to mitochondrial transplantation are rapid and enduring. Changes in transcriptomics are evident at 2 h and remain upregulated for at least 28 days, the extent of our current experimental recovery duration in our animal studies. Transcriptomic and proteomic changes occur rapidly and persist for at least 28 days.

9. Conclusion

The uses for mitochondrial transplantation are increasing. Mitochondrial transplantation has been used in the heart, lung, kidney, liver, skeletal muscle, brain and the eye (**Figure 4**). The importance of mitochondria as a therapeutic target and mitochondrial transplantation as a therapeutic modality is

evident and is expanding to involve a myriad of pathologies. Specific usage and procedural indices have been suggested and further modification and elucidation will occur. The data to date suggests that mitochondrial transplantation may provide new and improved approaches to many pathologies and conditions. This review provides the observations obtained from our studies with those of others in the area and does not include the many variations now being proposed. We hope that mitochondrial transplantation as a methodology will continue to increase in usage and for disease and non-disease states and that this review will stimulate further investigation.

Author contributions

JM: Writing – original draft, Writing – review & editing. PD: Writing – review & editing. SE: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Novel portable hypothermic machine perfusion preservation device enhances cardiac viability of donated human hearts

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Introduction: Heart transplant remains the gold standard treatment for patients with advanced heart failure. However, the list of patients waiting for a heart transplant continues to increase. We have developed a portable hypothermic oxygenated machine perfusion device, the VP.S ENCORE®, to extend the allowable preservation time. The purpose of this study was to test the efficacy of the VP.S. ENCORE® using deceased donors derived hearts.
Methods: Hearts from brain-dead donors not utilized for transplant ($n = 11$) were offered for research from the Texas Organ Sharing Alliance (TOSA), South and Central Texas' Organ Procurement Organization (OPO) and were preserved in the VP.S ENCORE® for 4 ($n = 2$), 6 ($n = 3$), and 8 ($n = 3$) hours or were kept in static cold storage (SCS) ($n = 3$). After preservation, the hearts were placed in an isolated heart Langendorff model for reperfusion and evaluated for cardiac function.
Results: The mean donor age was 37.82 ± 12.67 with the youngest donor being 19 and the oldest donor being 58 years old. SCS hearts mean weight gain (%) was −1.4 ± 2.77, while perfused at 4 h was 5.6 ± 6.04, perfused at 6 h 2.1 ± 6.04, and 8 h was 7.2 ± 10.76. Venous and arterial lactate concentrations were less than 2.0 mmol/L across all perfused hearts. Left ventricular contractility (+dPdT, mmHg/s) for 4 h (1,214 ± 1,064), 6 (1,565 ± 141.3), and 8 h (1,331 ± 403.6) were within the range of healthy human heart function. Thus, not significant as compared to the SCS group (1,597 ± 342.2). However, the left ventricular relaxation (mmHg/s) was significant in 6-hour perfused heart ($p < 0.05$) as compared to SCS. Gene expression analysis of inflammation markers (IL-6, IL-1β) showed no significant differences between SCS and perfused hearts, but a 6-hour perfusion led to a downregulated expression of these markers.
Discussion: The results demonstrate that the VP.S ENCORE® device enhances cardiac viability and exhibits comparable cardiac function to a healthy heart. The implications of these findings suggest that the VP.S ENCORE® could introduce a new paradigm in the field of organ preservation, especially for marginal hearts.

KEYWORDS

organ preservation, hypothermic machine perfusion, cardiac grafts, heart transplant, VP.S ENCORE® preservation device, deceased DBD donors, prolonged preservation

Introduction

Heart transplants (HT) remain the gold standard treatment for patients with advanced heart disease (1). However, worldwide, there are simply not enough heart donors available to meet the demand (2). In the United States alone, about 20% of patients on the heart transplant waiting list die or become too sick to remain good transplant candidates (3). Approaches to decrease organ shortage include the use of extended-criteria donors (ECDs) or donation after circulatory death (DCD) as well as the emergence of ex-situ machine perfusion (MP) systems as an alternative to the standard of care, the static cold storage (4). It has been estimated that DCD heart transplantation can increase the heart donor pool by about 30% (5). According to the United Network for Organ Sharing (6), 2022 was a record-setting year with a significant increase in DCD heart transplants in the United States. In DCD transplantation, only two techniques can be used: direct procurement and ex-situ perfusion (DPP) using TransMedics OCS, the only approved DCD technology in the United States, and the normothermic regional *in situ* perfusion (NRP) (7). The latter has emerged as a cost-effective alternative with promising early patient outcomes and high organ recovery rates (8, 9). After blood flow restoration, normothermic regional perfusion is followed by the SCS or MP (8). There are two types of machine perfusion technologies: normothermic (NMP) and hypothermic (HMP). Although both of them have their advantages and disadvantages, nevertheless, hypothermic perfusion is deemed to have a safer profile in the event of system failure, as opposed to normothermic machine perfusion (NMP), which carries the risk of causing irreversible damage to the heart (10). While in hypothermic preservation, the organ is cooled to a more standard static cold storage (SCS) temperature ranging from 5 to 10°C, a range considered by the International Society for Heart and Lung Transplantation (ISHLT) as an optimal temperature for the heart (11). Further, NMP involves additional surgical and technical support and appropriate transport, inevitably resulting in a more expensive management cost. While HMP holds tremendous potential for cardiac transplantation, most perfusion devices are quite complex and expensive, requiring blood-based products to be added to the proprietary perfusion solutions. Here, we present data on the HMP of human donor-derived hearts using the VP.S ENCORE®, an innovative and simple-to-use cardiac preservation device, which shows promising preclinical results for prolonged cardiac graft preservation.

Methods

Human donor heart procurement

Heart retrieval was performed in a standard fashion through a median sternotomy. The heart was exposed, and the donor was systemically heparinized with 30,000 units of heparin. After sufficient time for heparin circulation, the aorta was cross-clamped and one liter of cold cardioplegia was administered to arrest the heart. The heart was decompressed through the inferior vena cava and left atrial appendage. The donor cardiectomy was completed, and the graft was immersed in a cold saline solution for preparation and inspection.

Ex vivo hypothermic machine perfusion (HMP) and static cold storage preservation

Human donated hearts were either preserved using the VP.S ENCORE® or were kept in static cold storage. After Plasma-Lyte A (1 L) flush, donor hearts in the HMP group were cannulated with aortic and superior vena cava cannula to collect perfusate samples from both arterial and venous ports via oxygen probes. The heart was lowered into the organ storage canister filled with cold (4°C) Belzer MPS® UW Machine Perfusion solution and the perfusion module was secured to the canister with clamps for a liquid-tight seal. The prepared device was lowered into a temperature-regulated vessel and maintained at a hypothermic temperature. The VP.S ENCORE® device is lightweight, portable, and simple to use. The technology is based on hypothermic oxygenated machine perfusion that combines electro-fluidics and mechano-elastic principles to recover the energy inherently stored in compressed oxygen to drive preservation fluid through the coronary arteries of hearts. Compressed oxygen supplies an oxygenator while simultaneously driving fluid through the system into the aorta utilizing a novel diaphragm pump which allows the compact nature of the device and eliminates the need to use roller or centrifugal pumps (Figure 1). During HMP, perfusion flow, pressure, and temperature were recorded at a two-hour interval. Hearts in the static cold storage group were submerged in 1 L of Belzer UW® Cold Storage Solution double-bagged in ice slush and placed in an ice cooler for around 4 h. The perfusate temperature, flow, and pressure were monitored continuously.

Metabolic and cardiac edema assessment

During HPM, perfusate samples were collected from arterial and venous ports 30 min post the start of perfusion and every two hours afterward. Perfusate samples were analyzed for different blood gases using an i-STAT 200 analyzer (Abbott Laboratories) and cartridges for CG8 (general blood gases), CG4 (lactate), creatinine kinase-muscle/brain (CK-MB) and cardiac TroponinI (cTnI). Lactate expression was measured from both arterial and venous samples, whereas CK-MB and cTnI expression was obtained from only venous samples. The difference in lactate expression was calculated by subtracting arterial values from venous. Cardiac edema was determined by measuring the weights of hearts prior to and after each preservation method.

Oxygen consumption during perfusion and reperfusion

Myocardial oxygen consumption (MVO2 mlO2/min/100 g) was measured during perfusion and reperfusion. In the latter

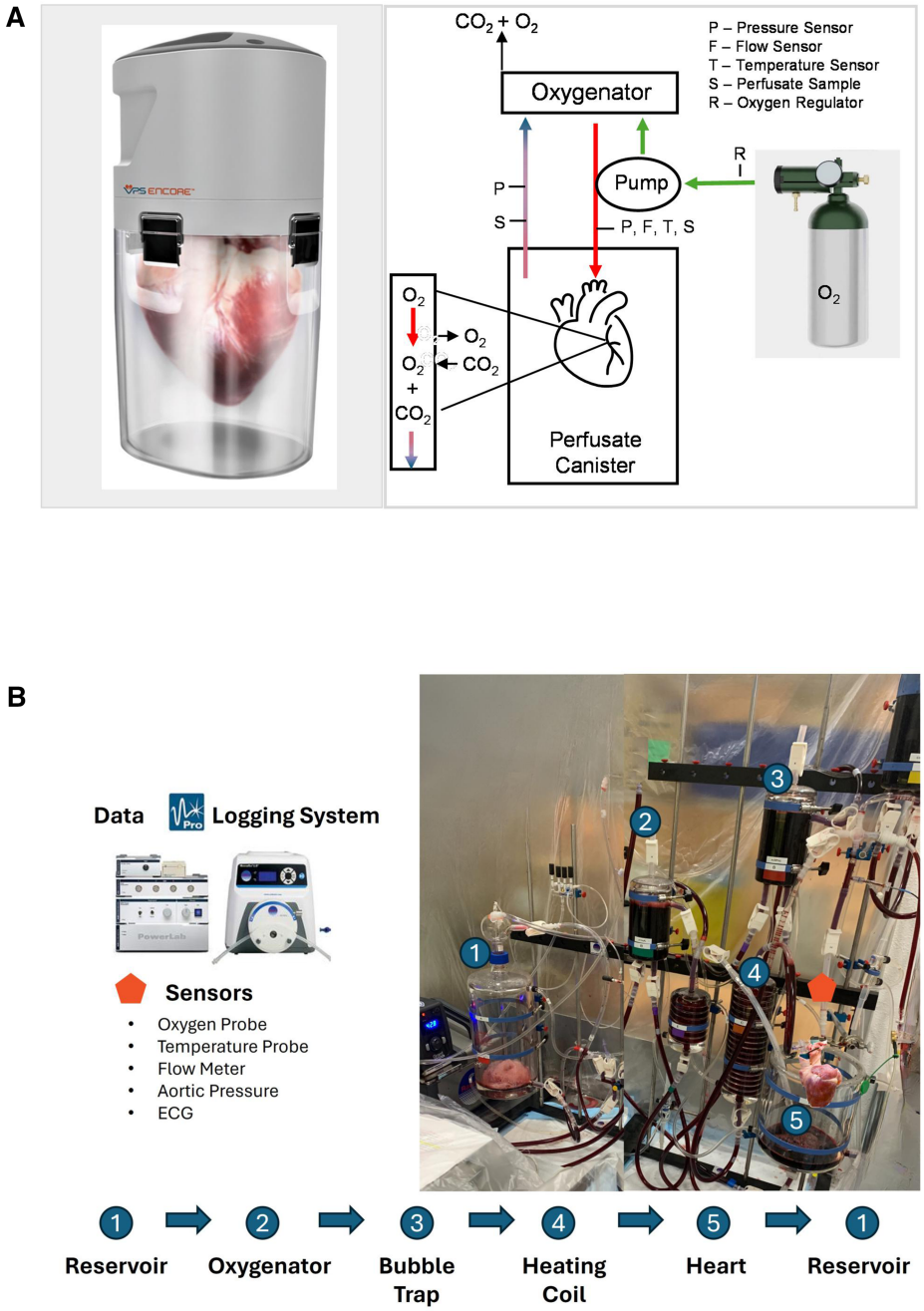

FIGURE 1
Schematic illustration of the (A) VP.S ENCORE® device. An external oxygen tank provides oxygen by pulsing at 50 pulses/min via a micro-solenoid at a fluid driving pressure of 10–20 mmHg. Oxygen travels both to the pump to drive fluid through the system and into the oxygenator to provide oxygen to the solution. The diaphragm pump directs oxygenated fluid directly to the aortic root. The perfusate provides oxygen and nutrients to the heart and fluid exits through the coronary sinus. The pressure gradient drives fluid from the canister and back into the head for reoxygenation. Deoxygenated fluid flows into the oxygenator and carbon dioxide and excess oxygen are vented into the atmosphere. This cycle continues throughout the entire preservation time. Perfusate samples are taken from a cannulated coronary sinus and from the arterial line. The arterial line has sensors to measure pressure, flow, and temperature. A second pressure sensor measures the pressure in the canister, and (B) schematic representation of the reperfusion on the Langendorff system where the numbers indicate the major components. To initiate a process, a flush solution consisting of 3l of modified Krebs buffer is circulated through the system using a peristaltic pump. This solution traverses through an oxygenator and bubble trap before entering the aorta. Subsequently, the flush solution is directed to waste. Once the temperature reaches 30°C, the solution is then switched to the main solution containing HBOC. As the heart gradually warms to 39°C, the perfusion flow rate is adjusted accordingly to meet the escalating oxygen demands, facilitating the process of stabilization. Different sensors are instrumented to collect reperfusion parameters, and the data has been continuously recorded using the Data Logging System.

phase, oxygen consumption was measured throughout an initial 30 min of the rewarming stage (before defibrillation) and after defibrillation. Two oxygen probes (Pyro FireSting^{TM}) collected oxygen partial pressure (mmHg) data of the solution before entering the coronary arteries (arterial) and after exiting the cannulated pulmonary artery (venous), whereas flow was

measured using the Sensirion flow meter. Oxygen consumption was captured using the following formula, ((([O2] a- [O2] v) * Q)/heart weight) * 100. [O2] a and [O2] v are defined as the oxygen content of arterial and venous perfusate respectively. [O2] was calculated as (1.34 * Hb* SO2) + (K * pO2); where 1.34 is ml O2/Hb (g), Hb is the concentration of hemoglobin measured in g/dl, SO2 is the oxygen saturation ratio calculated using the equation developed by Severinghaus (12), where K is the oxygen solubility coefficient adjusted for the perfusate temperature at every data point, pO2 is the partial pressure of oxygen in mmHg for the perfusate sample, and Q is a coronary flow in mL/min.

Assessment of cardiac function

After preservation, hearts were placed on a Langendorff system with a perfusate mixture of Krebs-Henseleit buffer, PEG-20kD, and hemoglobin-based oxygen carriers (HBOCs). The left ventricular function was expressed as the rate of pressure change over time dP/dT (mmHg/sec) and was measured by placing a pressure catheter (Millar, 5F) in the left ventricle. Data was analyzed using PowerLab (LabChart 8.1.16) blood pressure analyzer. Data was selected from the left ventricular pressure waveform as an average of 30 beats after stabilization (approximately 30 min after defibrillation). Contractility after ionotropic support was measured less than 5 min after administration of epinephrine (0.5 mg injected arterial). The end-diastolic pressure of the left ventricle was maintained by administering perfusate directly into the left ventricle through the mitral valve and venting to the atmosphere.

Myocardial histological assessment

Tissue biopsies (2.5–4 mm) were obtained pre and post-preservation from left and right ventricle vasculature for histological evaluation. Biopsies were placed in 10% formalin for 24 h and then stored for 3 days in 70% ethanol and embedded in paraffin. Samples were sectioned into 5 µm slides, stained with hematoxylin and eosin (H&E), and were scanned by Amscope microscope-scanner at 20× magnification. Myocardial injury was assessed based on the presence and severity of myofiber necrosis and degeneration, hemorrhage, interstitial edema, endothelial changes, and acute inflammation (13) and it was graded in a semiquantitative scale by an independent, blinded pathologist. Additionally, a subset of the hearts was sent to the cardiac pathology laboratory at the Texas Heart Institute for gross and microscopic evaluation by a blinded certified pathologist.

Gene expression

Total RNA from cardiac tissue biopsies was extracted following the TRIzol RNA isolation method. The concentration of total RNA was evaluated and measured at 260/280 nm by spectrophotometer (NanoVue Plus, GE Healthcare). Synthesis of cDNA was performed from 0.25 µg of total RNA, which was reverse transcribed using (iScript cDNA Synthesis Kit, Bio-Rad) according to the manufacturer's instructions. Gene-specific pre-designed oligonucleotide primers were purchased from Sigma. qRT-PCR was done using SsoAdvanced Universal SYBR Green Supermix and SFX96 Touch real-time PCR detection system (Bio-Rad, T100 Thermal Cycler). The cycling parameters were as follows: initial denaturation 95°C, 2 min; denaturation 95°C, 5 s; annealing/extension 60°C, 30 s; number of cycles 40; melt curve 65°–95°C (0.5°C increments). The comparative CT (2-ΔΔCT) method was used for all quantification. Values were normalized to the housekeeping gene.

Statistical analysis

All graphing and statistical analyses were performed using Prism 9 (GraphPad Software Inc., La Jolla, CA, USA). Results were expressed as means ± standard deviation (Std) or means ± standard error of the mean (SEM). Graphs were presented as overall means ± standard deviations/errors. Differences between the groups were assessed using a two-tailed Student's *T*-test for unpaired data. Statistical tests and corrections for multiple comparisons are described in each figure panel.

Results

Donor characteristics and reasons for transplant rejection

To avoid any potential bias, donor hearts were allocated into different preservation groups randomly. Most donor hearts have derived from females (60%) with no significant differences detected among other characteristics prior to heart procurement, such as donor age, ejection fraction, body max index (BMI), and sex (Figure 2). Further, human hearts were not utilized for transplant due to many different reasons, including donor age ($n = 2$), organ size ($n = 1$), rapid recovery ($n = 1$), unacceptable

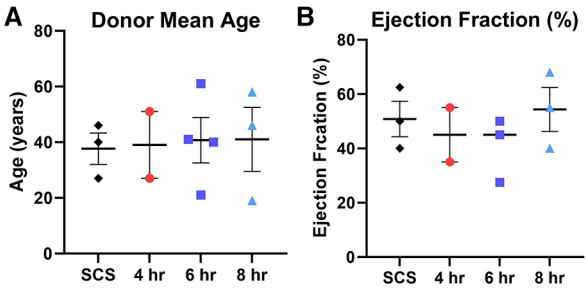

FIGURE 2
Donor characteristics prior heart procurement based on the preservation group including (A) donor mean age, and (B) donor ejection fraction (%). Each color represents a different preservation group, while each point represents an individual heart. Values plotted as means ± SEM. Graphs depicting age and ejection fraction of donors exhibit a distribution of data points with indicators for the mean, lowest, and highest values.

organ-specific test ($n = 1$), organ anatomical defect ($n = 1$), or due to no match run/medical rule out ($n = 5$). Drug overdose was the main cause of death for most donors ($n = 4$), while other causes were accidents, stroke, trauma, sepsis, or unknown reasons (Table 1).

Preservation parameters during HMP and static cold storage (SCS)

Human donor hearts were offered for research by the local procurement organization within a very short distance. We calculated both the time it took from the cross-clamp (CC) to start preservation as well as the total preservation duration (Figure 3A). Our data show no significant difference in cc—preservation time among different groups as well as no profound differences in perfusion parameters. The average coronary flow for all groups combined was 41.86 ± 5.09 ml/min with an average flow not exceeding 50 mL/min for each group individually. The average perfusion pressure recorded 12.61 ± 4.04 mmHg, and the average temperature was 7.99 ± 1.5°C (Figures 3B-D). Thus, every heart was assessed for edema by weighing the heart prior to and post-preservation. Our results show no significant change in weight gain (%) following any preservation method (See Figure 3E).

Biochemical markers of myocardial injury: lactates, CK-MB, and cardiac troponin I

Lactate was measured from the coronary sinus and arterial samples to determine the tissue anaerobic state. Study results show lactate expression not exceeding 2 mmol/L across all groups (Figure 4). The lowest expression was detected in the 4-hour perfusion group with arterial lactate measured at 0.31 ± 0.02 and venous 0.32 ± 0.02 mmol/L. The 6-hour perfusion group resulted in arterial lactate concentration of 1.72 ± 0.19, while venous expression was 1.95 ± 0.22 mmol/L, and the 8-hour group had arterial lactate expression of 0.95 ± 0.06, while venous had 0.92 ± 0.04 mmol/L indicating a shift from lactate production to lactate consumption. Further, the negative lactate difference (venous—arterial) was observed in a 6-hour preservation group which may indicate a higher level of lactate clearance or lower lactate production in the venous circulation compared to the arterial circulation. This could be indicative of improved tissue perfusion and oxygenation, which are critical factors in the preservation of donor hearts. Next, we assessed the expression of one of the creatine phosphokinases (CPK) isoenzymes, creatine kinase myocardial band (CK-MB), which is commonly obtained after heart transplantation (HT) as an indicator of a myocardial injury of the donor heart (14) (Figures 5A–B). Another common marker for cardiac cell damage is cardiac troponins. The specific isoform I (cTnI) is commonly detected in heart transplant recipients as an indicator of graft failure (15). There was no significant difference detected of cTnI expression among different perfusion groups with 4-hour perfusion resulting in 2.32 ± 2.09 ng/mL, while 6 and 8-hour perfusion groups resulting in 4.60 ± 3.87 and 3.08 ± 2.81 ng/mL respectively (Figures 5C–D). Due to insufficient research on assessing CK-MB and cTnI expression in human hearts during HMP, drawing conclusions about the levels of these markers' is challenging. Yet, when focusing on fold change over total concentration, a consistent fold change of 3.2 was found in all groups for CK-MB expression. In contrast, the 6-hour preservation group exhibited the lowest fold change in cTnI expression at 3.96 ± 0.44.

TABLE 1 Donor demographics and reasons for transplant rejection.

Donor characteristics				
Preservation group	SCS ($n = 3$)	4 h ($n = 2$)	6 h ($n = 3$)	8 h ($n = 3$)
Age	37.67 ± 9.71	39.00 ± 16.97	34.00 ± 11.27	41.00 ± 19.97
Ejection fraction (%)	50.83 ± 11.27	45.00 ± 14.14	40.83 ± 11.81	54.33 ± 14.01
BMI	20.70 ± 6.16	23.30 ± 1.28	24.77 ± 3.32	27.23 ± 4.29
Sex (M/F)	2/1	1/1	2/1	0/3
Rejection for transplant				
Donor age		1		1
Organ size				1
Rapid recovery		1		
Unacceptable organ specific test	1			
Organ anatomical defect	1			
No match run/ medical rule out	1		3	1
Cause of death				
Drug intoxication	1	1	2	
Accidents				1
Stroke	2		1	
Trauma		1		
Sepsis				1
Uknown				1

Myocardial oxygen consumption

Myocardial oxygen consumption correlates with energy utilization from the cells and is considered a good marker to assess energy production and uptake by the myocardial tissue (16, 17). In this study, we assessed oxygen consumption during the perfusion and reperfusion phases (Figure 6). The average oxygen consumption for 4-hour preservation during HMP was 0.70 ± 0.1 mlO_2/min/100 g, while for 6 and 8-hour preservation was 0.50 ± 0.09 and 0.23 ± 0.05 respectively. Hearts in different preservation groups show similar oxygen consumption trends which fall under the error band area representing the 95% confidence interval. Thus, our data is in alignment with findings from other investigators (18, 19). Oxygen consumption during the re-reperfusion stage shows normal oxygen consumption ranging from 2 to 5 mlO2/min/100 g across all groups (20).

Assessment of cardiac function

After preservation, hearts were removed from the VP.S ENCORE® and placed in a Langendorff system for reperfusion and evaluation of cardiac contractility. This system assesses left ventricular dP/dT without preload and afterload, thereby allowing the evaluation of the intrinsic contractility of the left

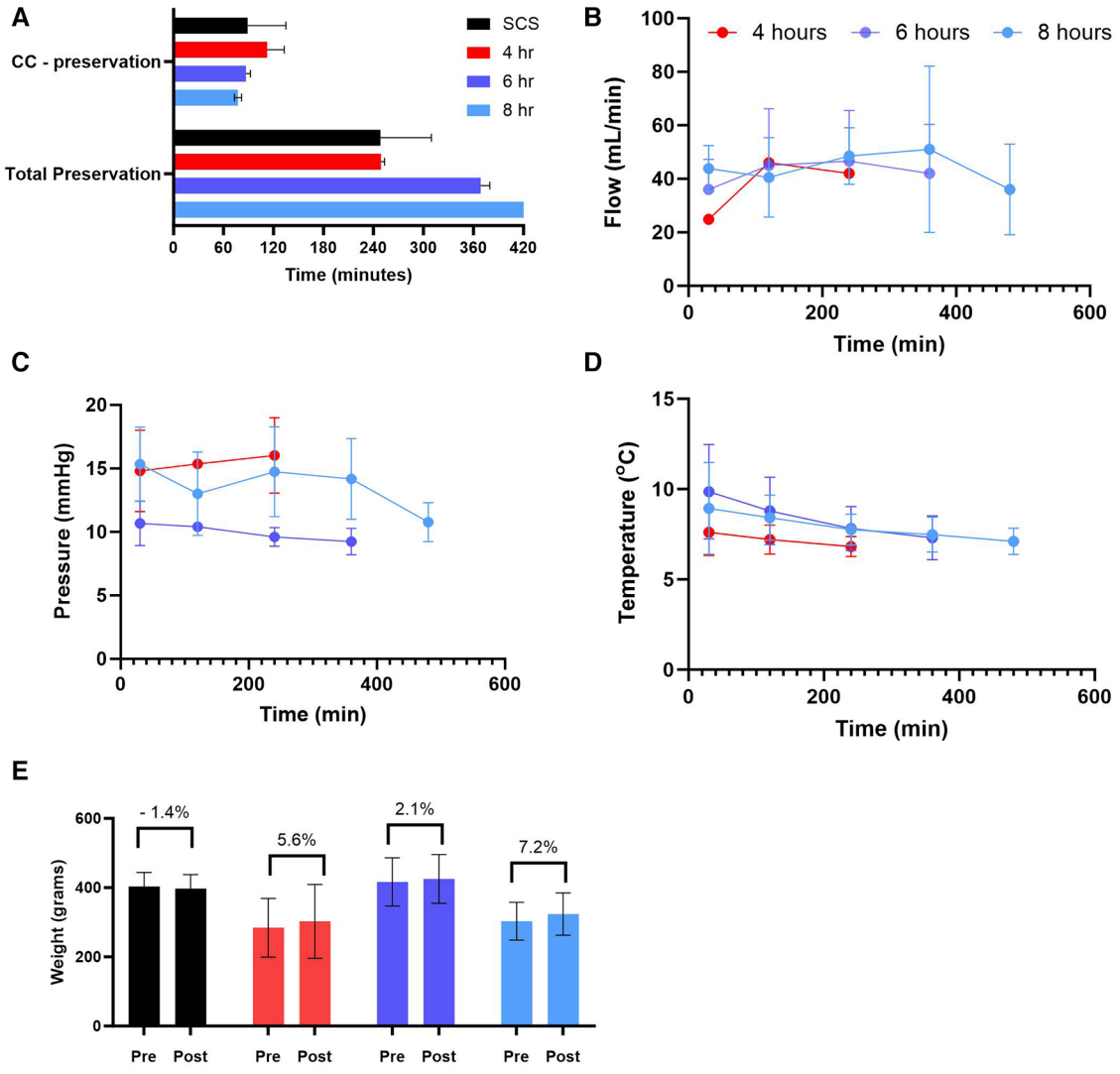

FIGURE 3
Preservation parameters during static cold storage (SCS) and hypothermic machine perfusion (HMP). (**A**) time (5) of cross-clamp (CC) to the start of preservation and total preservation time, (**B**) coronary flow during HMP, (**C**) perfusion pressure during HMP, (**D**) perfusion temperature during HMP, and (**E**) weight gain calculated post preservation for each group. Data expressed as means ± SEM.

ventricle. No substantial change in left ventricle (3) contractility was detected among different groups with all of them corresponding to a normal range > 1,200 mmHg/s (Figure 7A) (21). However, there was a significant ($p < 0.05$) increase in LV relaxation in hearts perfused for 6 h (Figure 7B) and no significant difference in LV maximum pressure (Figure 7C).

Myocardial histological assessment

Representative histological images from hearts collected pre and post-preservation from the apex area showed no obvious ventricular differences within each heart (Figure 8A). Myocardial histological assessment post-preservation, characterized by the myocardial injury score, revealed a significant ($p < 0.04$) reduction in myofiber degeneration in the 4-hour preservation hearts group compared to the SCS group as characterized by the presence of leaky nuclei. There were no significant differences in other myocardial assessment parameters (myocardial hemorrhage, interstitial edema, and endothelial changes) between the standard of care and HMP groups.

Gene expression analysis

Gene expression analysis of inflammation markers interleukin (IL)-6 and interleukin (Il)-1β assessed in tissues collected at the end of each preservation method, showed no significant difference among different groups (Figure 9). However, some groups had profound downregulation of these inflammation markers, although not significant. As such, 6-hour perfusion led to a 45% downregulated expression of IL-6 and a 74% downregulation of IL-1β as visualized by the median dotted line in violin plots. Also, 4-hour perfusion led to a 34% downregulated expression of IL-1β. Elevated levels of these markers are known to be associated with

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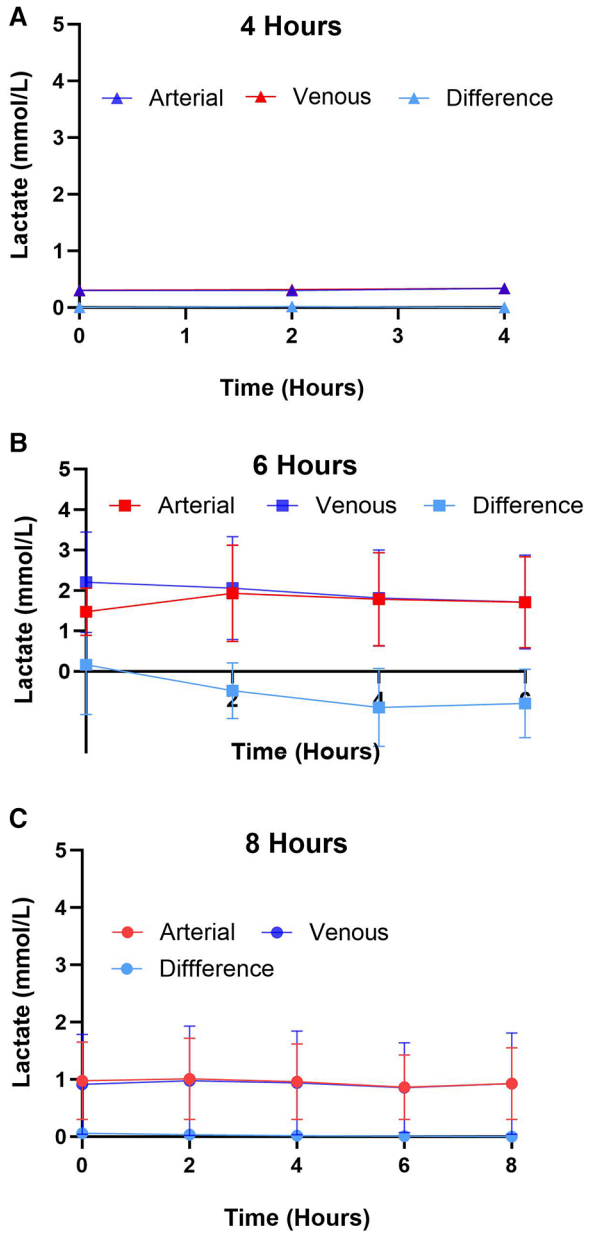

FIGURE 4
Arterial and venous lactate expression for different preservation groups including (A) 4 h, (B) 6 h, and (C) 8 h during hypothermic preservation. The difference in lactate expression, calculated as the arterial lactate concentration minus the venous lactate concentration, is also depicted on the graph. This provides insights into the net production or consumption of lactate by the heart during hypothermic perfusion. Samples were collected every two hours, and data displayed as means ± Std.

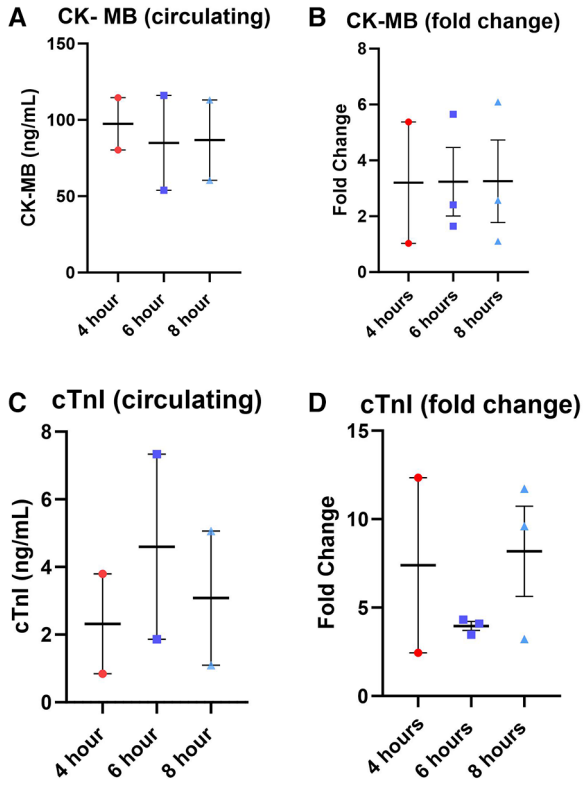

FIGURE 5
Total expression and fold change of circulating markers creatine kinase MB (CK-MB) and cardiac troponin I (cTnI) across all perfusion groups. (A) total expression of CK-MB followed by (B) the fold change, total expression of cTnI (C), and (D) the fold change. Values were obtained from venous samples at the beginning and at the end of perfusion. The fold change is calculated by dividing the expression level at the end by the baseline expression level. Each point represents an individual heart with indicators for the mean, lowest, and highest values. Data expressed as means ± SEM.

inflammatory responses, cardiac remodeling, and heart failure (22, 23). However, their precise role in HMP and implications for clinical outcomes are yet to be delineated in future studies.

Discussion

The growing demand for heart transplants remains a significant challenge, outpacing the availability of suitable organs, which is largely attributed to improved overall survival post-myocardial infarction and the aging global population (2). The availability of suitable hearts for transplantation continues as a major limit on the growing demand for heart transplantation. The primary component of this limitation is the 4-hour cross-clamp to cross-clamp preservation time imposed by the current standard of care, static cold storage. Each hour exceeding 4 h increases the probability of delayed graft function and primary graft failure leading to poorer morbidity and mortality (24, 25). As such, only hearts recovered within 4 h of the transplant site are typically accepted with a large proportion of suitable hearts being left unclaimed. Addressing this organ shortage is crucial, and machine perfusion technology, particularly when it is accessible and user-friendly, holds promise as a key strategy to enhance heart transplant utilization rates both in the United States and globally. The VP.S ENCORE®, a single use-device, weighing less than 23 Kg (50 pounds), and may be accompanied by only one technician with very minimal training (<30 min).

This study aimed to evaluate the feasibility of the VP.S ENCORE® device to preserve human donor-derived hearts not

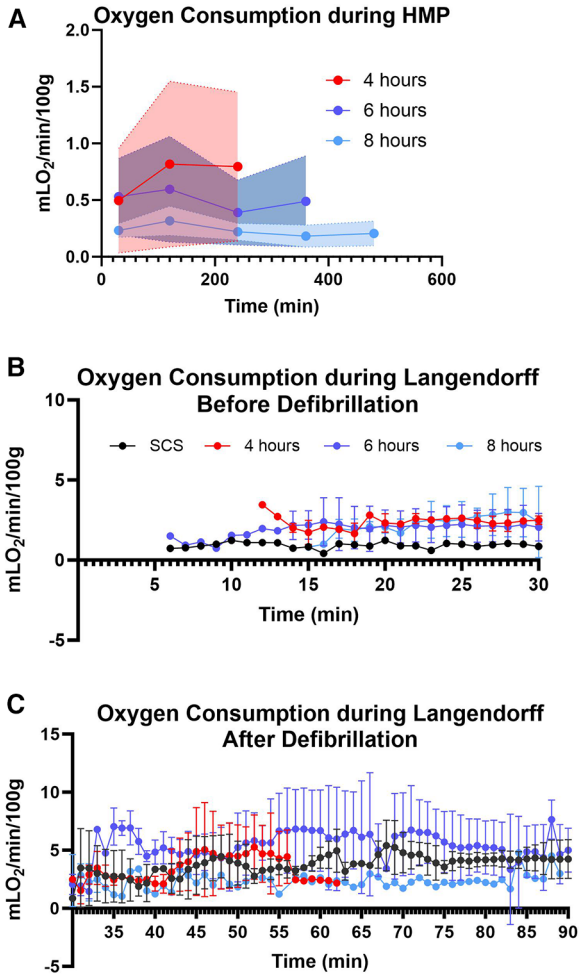

FIGURE 6
Myocardial oxygen consumption. (A) during hypothermic machine perfusion measured every two hours, (B) during reperfusion on the Landendorff system before defibrillation, and (C) on the Langendorff after defibrillation measured every 5 min. Shaded colors represent the error band area for each preservation group under the 95% confidence interval. Data displayed as means ± Std.

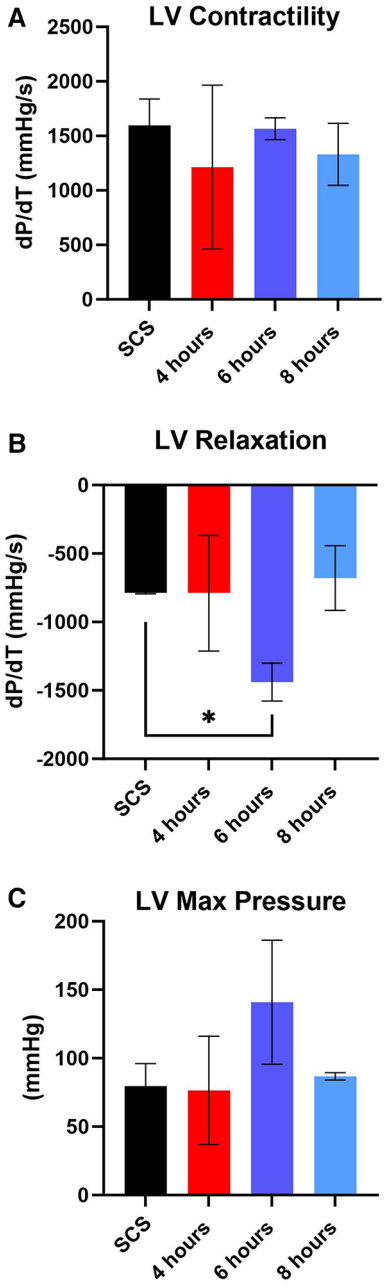

FIGURE 7
Assessment of left ventricular (LV) cardiac function during reperfusion. (A) LV contractility, (B) LV relaxation, and (C) LV maximum pressure. Data displayed as means ± SEM. Significance ($p < 0.05$) was determined by using the *t*-test.

utilized for transplant for prolonged time. Study results revealed that hypothermic machine perfusion (HMP) between 4 and 10°C contributes to the reduction of the active metabolic state in cardiac grafts, as measured by low lactate and other myocardial markers expression, thereby minimizing the risk of the accumulation of toxic metabolites while maintaining cardiac viability (26). Examination of various metabolic markers, including oxygen consumption and lactate levels, demonstrated notable correlations with myocardial function during machine perfusion (4). The Arrhenius equation can be used to estimate the organ's oxygen demand during cardiac preservation. This equation is relevant to understanding the metabolic characteristics of human hearts preserved for a specific duration, and it provides insights into the rate of cellular processes and cardiac mitochondrial protection (27). Results indicate that the oxygen consumption during HMP was consistent with the Arrhenius prediction at 7°C for each of the groups and the results demonstrate that the VP.S ENCORE® delivered more than adequate levels of oxygen to the myocardium to cover its metabolic demand. Oxygen consumption prior to and post-defibrillation on the Langendorf was comparable in all groups and within the confidence interval predicted by the Arrhenius Equation at 37°C. Notably, study findings align with, and support results reported by other studies, reinforcing the consistency and significance of our observations. Additionally, the low perfusion pressures (<20 mmHg) minimized hydrostatic edema development to less than 8%. While the extent of the

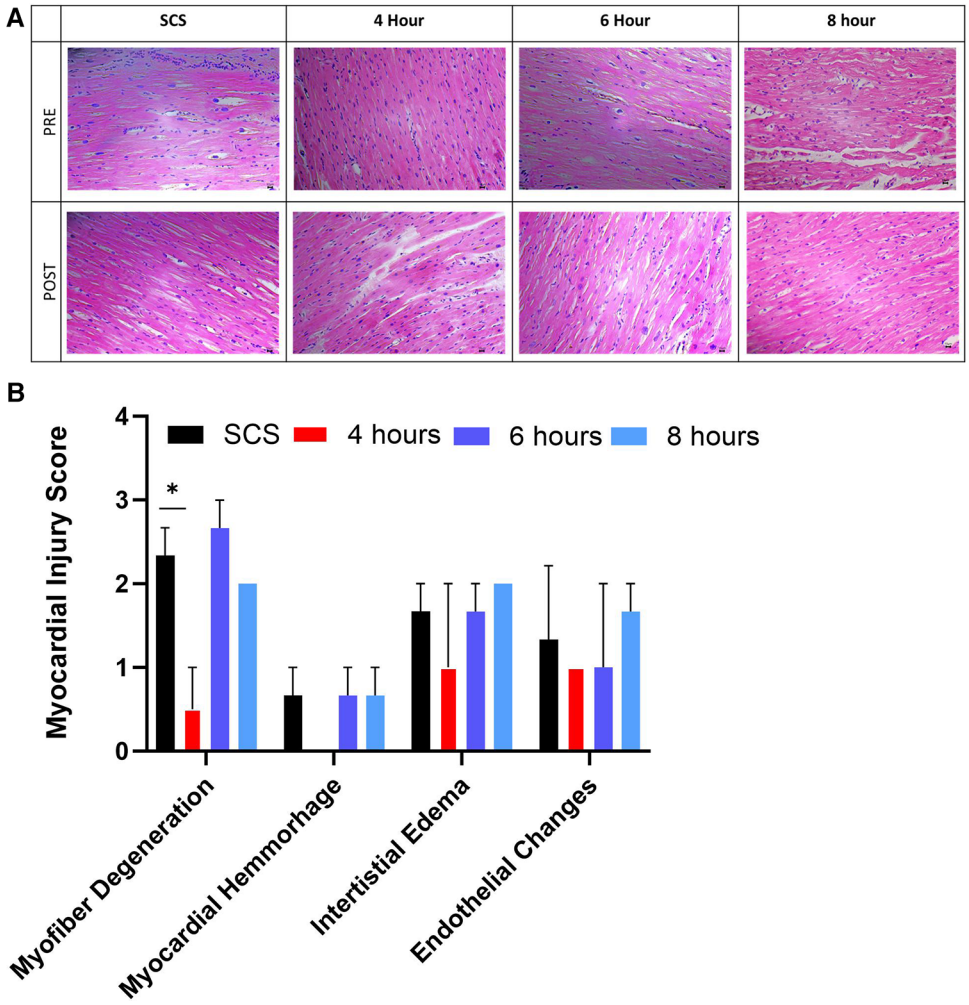

FIGURE 8
Myocardial histological assessment. (A) representative histological images from the apex area of hearts collected pre and post-preservation, followed by (B) myocardial injury score assigned by blinded pathologists. Myocardial injury score ranges from 0 to 3, where 0 is none, while 3 is severe. Data displayed as means ± Std. Significance ($p < 0.05$) was determined by using the t-test.

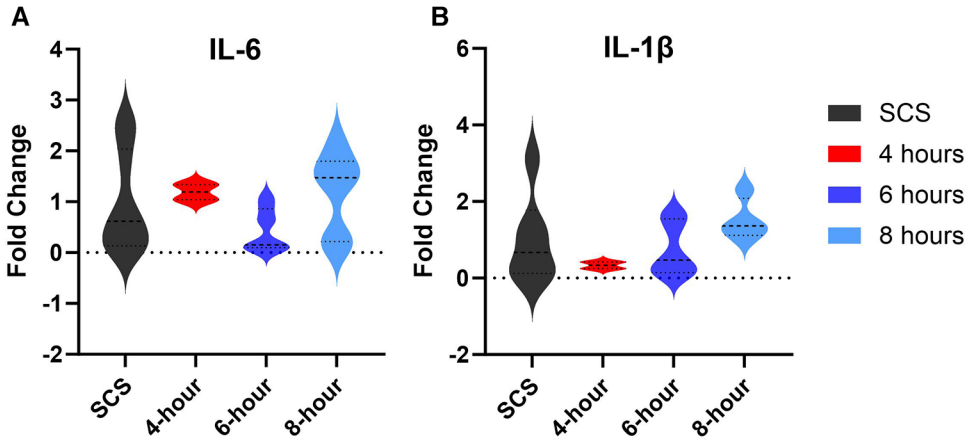

FIGURE 9
Gene expression of tissue inflammation markers. (A) IL-6 expression, (B) IL-1β expression. Tissues were obtained from the apex area after preservation and the fold change was calculated by the qRT-PCR. Data displayed as means ± Std and presented as violin plots with width representing the density of the data, and the shape showing the distribution of the data.

edema may not be the only factor in determining the suitability for transplantation, severe edema can impact the success of organ transplantation (28). One of the advantages of the VP.S ENCORE® device is no need for blood or blood-based products to be used. The solution of choice in this study was the Belzer MPS® UW Machine Perfusion solution, designed to keep the heart in diastolic arrest and to slow down cellular metabolism, reduce edema, and maintain cell integrity (17). While left ventricular contractility in the perfusion groups was similar to the SCS control, left ventricular relaxation following 6 h of perfusion was significantly greater. These findings are of particular significance in that cardiac function appears to have been maintained after an extended time of preservation. In addition, myocardial histological assessment and lower inflammation markers gene expression are consistent with the oxygen consumption and contractility data further reinforcing the notion that VP.S ENCORE® provides a superior preservation environment for cardiac grafts.

Despite these promising outcomes, it is essential to acknowledge the limitations of this study. Further testing and validation, particularly in larger cohorts and diverse clinical scenarios, are necessary to establish the generalizability and robustness of these findings. Additionally, the long-term effects of the VP.S ENCORE® device and its compatibility with various donor types warrant thorough exploration before widespread clinical application. The complexities and variability associated with human hearts, especially those procured from donation after circulatory death (DCD) donors, underscore the need for continued research and refinement of machine perfusion technologies. Preliminary data collected on using the VP.S ENCORE® to preserve DCD porcine hearts (data not shown) presents a promising avenue for the preservation of such hearts, potentially revolutionizing organ transplantation practices. Through controlled hypothermic perfusion and sophisticated monitoring capabilities, the VP.S ENCORE® device aims to mitigate ischemic injury, maintain metabolic homeostasis, and minimize the risk of organ rejection post-transplantation. Furthermore, the VP.S ENCORE® device may offer flexibility in transportation logistics and extend the window of opportunity for organ retrieval, enhancing accessibility to viable organs for transplantation. As ongoing research continues to explore its efficacy and refine its functionalities, the VP.S ENCORE® emerges as a promising tool in addressing the challenges associated with DCD heart preservation and advancing the field of organ transplantation.

The current study contributes valuable insights into the potential of the VP.S ENCORE® cardiac preservation device as a means to address the ongoing heart utilization challenges in heart transplantation. By building upon established principles of hypothermic machine perfusion and exploring innovative applications such as gene therapy and xenotransplantation, this research lays the groundwork for future advancements in organ preservation and transplantation strategies. While acknowledging the need for further investigation and refinement, the encouraging findings from this study emphasize the role of machine perfusion technology in shaping the future landscape of heart transplantation.

Conclusions

The study's results demonstrate that human-derived donor hearts preserved in the VP.S ENCORE® for a prolonged time (4–8 h) had comparable and in most cases better outcomes than hearts stored using the standard of care preservation method. Given the hearts used in this study were rejected for transplant and had an initial lower ejection fraction (40%–50%) than typically accepted for transplantation, study findings suggest that the VP.S ENCORE® holds promise in preserving not only standard criteria but also "marginal" donor hearts, potentially further amplifying the utilization rates of heart transplants. Extending heart preservation time has the potential to improve organ allocation, increase transplant success rates, and enhance the overall efficiency of organ transplantation systems. However, careful consideration of associated challenges and risks is essential in implementing and optimizing these practices.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author.

Ethics statement

The studies were conducted in accordance with the local legislation and institutional requirements. Ethical approval was not required as the human hearts were collected from deceased donors upon permission from donors' families through the agreement with the Texas Organ Sharing Alliance (TOSA), South and Central Texas' Organ Procurement Organization (OPO).

Author contributions

KA: Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Project administration, Methodology, Investigation, Formal Analysis, Data curation, Conceptualization. RV: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. RL: Writing – review & editing, Software, Methodology, Investigation, Data curation. ZM: Writing – review & editing, Supervision, Methodology, Formal Analysis, Data curation. IC: Writing – review & editing, Software, Methodology, Investigation. EC: Writing – review & editing, Software, Methodology, Investigation, Data curation, Conceptualization. IJ: Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. MB: Writing – review & editing, Methodology, Investigation, Software. GL: Writing – review & editing, Methodology, Supervision. MW: Writing – review & editing, Methodology, Investigation, Formal Analysis,

Conceptualization, Visualization, Validation, Supervision, Software, Resources, Project administration, Funding acquisition, Data curation. JN: Writing – review & editing, Resources, Methodology, Investigation. MO: Methodology, Investigation, Writing – review & editing, Resources. LB: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization.

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Conflict of interest

KA, RV, RL, ZM, IC, EC, IJ, MB, GL, MW, and LB are employees at the Vascular Perfusion Solutions, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Extending heart preservation to 24 h with normothermic perfusion

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Cold static storage (CSS) for up to 6 h is the gold standard in heart preservation. Although some hearts stored over 6 h have been transplanted, longer CSS times have increased posttransplant morbimortality. Transmedics® Organ Care System (OCS™) is the only FDA-approved commercial system that provides an alternative to CSS using normothermic *ex situ* heart perfusion (NEHP) in resting mode with aortic perfusion (Langendorff method). However, it is also limited to 6 h and lacks an objective assessment of cardiac function. Developing a system that can perfuse hearts under NEHP conditions for >24 h can facilitate organ rehabilitation, expansion of the donor pool, and objective functional evaluation. The Extracorporeal Life Support Laboratory at the University of Michigan has worked to prolong NEHP to >24 h with an objective assessment of heart viability during NEHP. An NEHP system was developed for aortic (Langendorff) perfusion using a blood-derived perfusate (leukocyte/thrombocyte-depleted blood). Porcine hearts ($n = 42$) of different sizes (6–55 kg) were divided into five groups and studied during 24 h NEHP with various interventions in three piglets (small-size) heart groups: (1) Control NEHP without interventions ($n = 15$); (2) NEHP + plasma exchange ($n = 5$); (3) NEHP + hemofiltration ($n = 10$) and two adult-size (juvenile pigs) heart groups (to demonstrate the support of larger hearts); (4) NEHP + hemofiltration ($n = 5$); and (5) NEHP with intermittent left atrial (iLA) perfusion ($n = 7$). All hearts with NEHP + interventions ($n = 27$) were successfully perfused for 24 h, whereas 14 (93.3%) control hearts failed between 10 and 21 h, and 1 control heart (6.6%) lasted 24 h. Hearts in the piglet hemofiltration and plasma exchange groups performed better than those in the control group. The larger hearts in the iLA perfusion group ($n = 7$) allowed for real-time heart functional assessment and remained stable throughout the 24 h of NEHP. These results demonstrate that heart preservation for 24 h is feasible with our NEHP perfusion technique. Increasing the preservation period beyond 24 h, infection control, and nutritional support all need optimization. This proves the concept that NEHP has the potential to increase the organ pool by (1) considering previously discarded hearts; (2) performing an objective assessment of heart function; (3) increasing the donor/recipient distance; and (4) developing heart-specific perfusion therapies.

KEYWORDS

heart preservation, normothermic, *ex situ*, *ex vivo*, prolonged, perfusion, extracorporeal support

Introduction

Cold static storage (CSS) for up to 6 h has historically been the gold standard in heart preservation. Although some hearts stored over 6 h have been transplanted, longer CSS times have increased posttransplant morbimortality secondary to cold ischemia, endothelial damage, and loss of vasomotor tone. Recently, there has been increased research on normothermic *ex situ* heart perfusion (NEHP) for prolonged storage of heart tissues prior to transplantation.

Transmedics Organ Care System (OCS^{TM}) is the only FDA-approved commercial perfusion system that provides an alternative to CSS using NEHP in resting mode with aortic perfusion at the rate of coronary perfusion (Langendorff method). However, it is also limited to 6 h and lacks an objective assessment of cardiac function.

Over the past 7 years, the Extracorporeal Life Support Laboratory at the University of Michigan has worked to prolong NEHP. We demonstrated that hearts could be routinely perfused and preserved for 3 days when an aliquot of plasma was continuously exchanged between the blood perfusate and a live paracorporeal animal (sheep). Something is removed and/or added that allows long-term successful NEHP. These factors are humoral (not cellular) and present in plasma (1). We conducted a series of experiments to identify those factors without the paracorporeal animal. This was done in a stepwise fashion beginning with fresh donor plasma exchange for 24 h in piglet (small-size) hearts (2). These experiments demonstrated that the critical factors are added/removed by plasma exchange alone. Next, we evaluated hemofiltration to assess the toxic factor removal component of plasma exchange. Hemofiltration at 1 cc/g/h allowed successful 24-h perfusion. We evaluated the filtrate to determine which critical molecules are removed. The only nutrition in this series of studies was glucose added in the filtration replacement fluid (3). All of these studies were done with an aortic perfusion of the coronary circulation at around 1 mL/min/g of cardiac tissue. Studies are ongoing to evaluate left atrial (working mode) perfusion to measure heart function during NEHP (publication pending). In this report, we summarize our published and ongoing experience with NEHP without a paracorporeal animal.

Methods

Animals

Forty-two healthy pigs (6–55 kg) were utilized during 24 h NEHP runs with various interventions. Piglet (small-size) hearts: (1) Control NEHP without interventions ($n = 15$); (2) NEHP + plasma exchange ($n = 5$); (3) NEHP + hemofiltration ($n = 10$). Juvenile pig (adult-size) hearts were used to prove the adequate support of larger hearts NEHP + hemofiltration ($n = 5$) and the assessment of heart function in real time while on NEHP with intermittent left atrial (iLA) perfusion ($n = 7$). All animals received humane care in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and protocols were approved by the University of Michigan Institutional Animal Care and Use Committee (Protocol # 11170, Approved 18 January 2023).

Surgical procedure

The surgical procedure was previously described by Tchouta et al. (2) and Johnson et al. (3) and is unchanged from prior experiments. A brief description is as follows. Isoflurane-inhaled general anesthesia was induced with ketamine–zolazepam (7 mg/kg) combined with xylazine (3 mg/kg). The skin was prepared and draped in a standard sterile manner, and intravenous antibiotics were administered (nafcillin 25 mg/kg and gentamicin 2.25 mg/kg). Lidocaine (1 mg/kg) was administered intravenously before midline sternotomy. The pericardium was left intact to minimize tissue desiccation. The extrapericardial great vessels were isolated and loosely encircled with ligatures. The animals received intravenous unfractionated heparin 400 IU/kg (Sagent Pharma, Schaumburg, IL, USA). Following documentation of adequate systemic anticoagulation, a cardioplegia needle was placed in the proximal innominate artery and the distal innominate and left subclavian arteries were ligated. The proximal intrathoracic inferior vena cava (IVC) and the left azygous vein were ligated, and the pig was exsanguinated through the distal IVC using a 20–24Fr cannula and standard sterile blood collection bags. Concomitantly, the mid-descending thoracic aorta was cross-clamped, the superior vena cava (SVC) was ligated, and 1 L of cold (5 °C) del Nido cardioplegia (CAPS Inc., Detroit, MI, USA) was subsequently administered through the proximal innominate artery. The left heart was decompressed by transecting the right pulmonary veins. Sterile saline ice slush was applied to the heart during cardioplegia administration. After the administration of cardioplegia and confirmation of cessation of heart function, the hearts were excised with the pericardium intact, weighed, and placed in an ice bath for back table preparation aiming for <60 min of cold ischemia time (CIT).

Back table preparation

A 10Fr venous drainage cannula (Medtronic, Minneapolis, MN, USA) and a customized high-compliance balloon, secured to the cannula (Dynarex, Orangeburg, NY, USA), connected to a pressure transducer apparatus, were inserted into the left ventricle (LV) to monitor LV pressure. A 5-0 polypropylene suture was placed into the posterior leaflet of the mitral valve and secured to the cannula to prevent balloon migration. The modified LV compliance balloon was used for all NEHP studies except for the ones where iLA perfusion was performed, as LV ejection was required. A 20Fr DLP venous drainage cannula (Medtronic Inc., Dublin, Ireland) was placed into the right ventricle (RV) via the pulmonary artery (PA). A customized 1/4 × 3/8-in connector with Luer Lock (Medtronic, Minneapolis, MN, USA) was secured in the aortic arch for antegrade coronary

perfusion (Langendorff). All remaining branches were ligated, including the SVC, IVC, subclavian artery, and innominate artery. The heart and cannulae were de-aired and connected to the NEHP system.

Circuit, circuit priming, and blood-derived perfusate

The perfusion circuit (Figure 1) consisted of a reservoir (Terumo, Ann Arbor, MI, USA), an FX05 Baby Capiox Oxygenator (Terumo CVS, Ann Arbor, MI, USA), and an Mpump (peristaltic-modified roller pump) (Daris LLC, Ann Arbor, MI, USA). The priming of the circuit contained several steps: (1) 400–500 mL of Plasma-lyte A (balanced crystalloid solution) to remove any air in the system; (2) Plasma-lyte A is removed from the system (as much as possible); and (3) addition of a blood-derived perfusate from the IV transfer bag (see below) into the NEHP reservoir.

Under general anesthesia, healthy pigs (100–120 kg) were exsanguinated and used as blood donors. Blood (5–6 L) was collected using the three-bag Teruflex® (Terumo Corp., Tokyo, Japan) blood bag system with citrate phosphate dextrose adenine solution (CPDA-1) anticoagulant. The volume of blood per kit was 450 mL. The collected blood was then stored in a 5 °C refrigerator for up to 10 days on a rocking system until its use. Whole blood was then separated using the collecting blood bags via centrifugation (Sorvall Legend XFR Centrifuge—Thermo Fisher Scientific, Waltham, MA, USA) for 20 min at 25 °C with 3,600 RPM. Plasma and packed red blood cells (pRBCs) were then collected using the plasma extractor and Fenwal transfer set (Fresenius Kabi AG, Bad Homburg, Germany), and the buffy coat (platelet and white blood cells) was discarded.

The priming volume was approximately 250–300 mL of platelet- and leukocyte-reduced blood with a hemoglobin (Hb) concentration goal >8 g/dL and hematocrit ≥24% for all studies. The blood-derived perfusate was then oxygenated and conditioned to normothermic conditions (37 °C) prior to connecting the heart to NEHP. Washed pRBCs from our animal blood bank were used to maintain Hb >8 g/dL. Normal saline was used to wash pRBCs if the K values were >9 mmol/L. In addition, calcium was monitored immediately after pRBC and hourly during NEHP and replenished if ionized Ca <1.1 mmol/L with 250 mg calcium gluconate (1 cc bolus). Glucose was replaced if perfusate levels were <60 mg/dL, but this situation rarely occurs during NEHP with the addition of plasma exchange or hemofiltration, unless contamination with bacteria is observed.

Perfusion protocol

Aortic blood flows were slowly increased and adjusted to maintain coronary blood flow between 0.5 and 1.0 mL/min/g of cardiac tissue (mL/min/g) with a mean flow of 0.7 mL/min/g concordant with physiologic coronary blood flow using Langendorff perfusion. PA outflow and LV drainage were collected and returned to the reservoir of the perfusion circuit. The LV compliance balloon was inflated to maintain initial end-diastolic pressure within 8–10 mmHg; the initial volume required to reach this pressure was kept consistent throughout the duration of the prep. PA venous saturations were targeted to 75%–90%, and coronary flow adjusted accordingly. The temperature was maintained at 37 °C using a water heater (CSZ Cincinnati Sub-Zero ECMO Heather, Cincinnati, OH, USA). The sweep gas (50% O_2, 45% N_2, and 5% CO_2) was adjusted to maintain pCO_2 at 40 ± 5 mmHg. If fibrillation occurred, the heart was defibrillated with 5–10 J using internal defibrillation paddles (Philips, Andover, MA, USA). The perfusate was exchanged at 60 min of NEHP to eliminate residual cardioplegia and toxins that may have accumulated from reperfusion. The

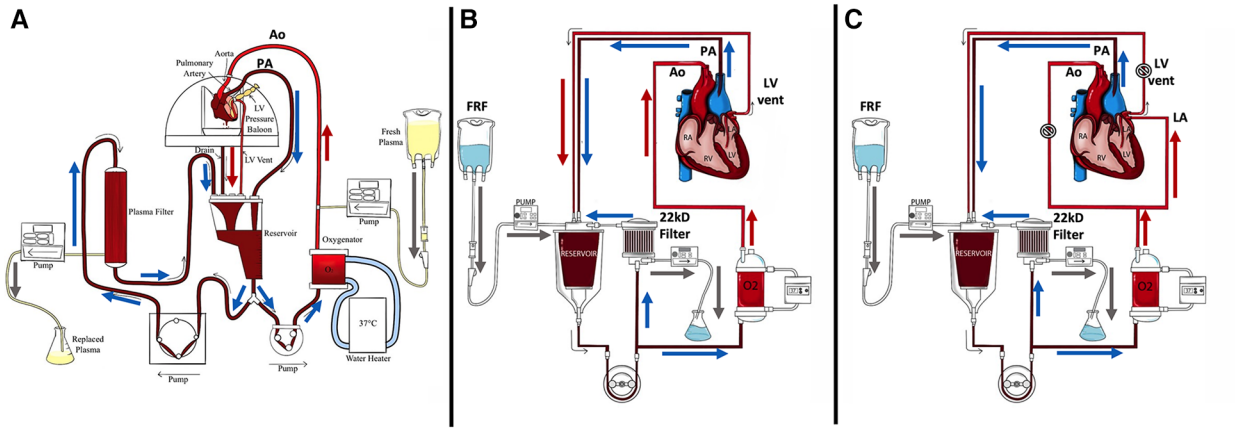

FIGURE 1
Diagrams of NEHP circuits. (**A**) An NEHP circuit used for pediatric hearts with plasma exchange (piglet hearts) using a plasma separator (Plasmaflo OP-05W[A] (Asahi Kasei Medical MT Corp., Oita, Japan); (**B**) An NEHP circuit used for pediatric (piglet) and adult (juvenile pigs) heart models with hemofiltration [Prismaflex HF1000 filter (22Kd) Baxter Inc., Deerfield, IL, USA]; and (**C**) NEHP circuit modifications for adult (juvenile pigs) heart iLA perfusion studies. Ao, aortic root line; FRF: Filtrate Replacement Fluid; LV: Left Ventricle; PA: Pulmonary Artery line. ⊘, clamping of line for iLA perfusion.

perfusate exchange followed cardiopulmonary bypass practices. The volume in the reservoir was depleted to a safe level (−30 cc), and the new perfusate was added while the outflow from the heart was collected and discarded. This maneuver mitigated air embolism, while most of the perfusate was exchanged without a stoppage of perfusion of the heart.

Summary of individual experiments

Plasma exchange

Plasma was infused into the aortic catheter in the experimental hearts and filtered out at a similar rate from the reservoir drainage line. The previous plasma cross-circulation experiments included removal of plasma from the perfusion circuit and return to the paracorporeal animal, creating continuous plasma exchange (2). In the current experiments, continuous plasma exchange was done by a continuous infusion of bank plasma and continuous removal of the same amount of plasma by a plasma separator [Plasmaflo OP-05W(A), Asahi Kasei Medical MT Corp., Oita, Japan] (Figure 1A).

Hemofiltration

Perfusion was performed in parallel with hemofiltration (3). The hemofilter was Prismaflex HF1000 (Baxter Inc., Deerfield, IL, USA), which filters molecules up to 22 kD. Perfusate hemofiltration was maintained at 1 mL/h/g using an IV pump. Isotonic filtrate replacement fluid (FRF) was added to the perfusate at a 1:1 ratio. One liter of FRF consisted of 750 mL 0.9% saline solution and 250 mL with 3.3 g of glucose, 400 mg calcium gluconate, 30 mEq bicarbonate, 160 mg magnesium, 4 mEq potassium, 250 mg nafcillin, and 40 mg of gentamicin (Figure 1B).

iLA perfusion

For the iLA perfusion, a 10Fr venous drainage cannula was inserted into the LV for LV drainage. A 20Fr DLP venous drainage cannula (Medtronic Inc., Dublin, Ireland) was placed into the RV via the PA. A 1/4 × 3/8-in connector with Luer Lock (Medtronic, Minneapolis, MN, USA) was secured in the aortic root for antegrade coronary perfusion. A 20Fr malleable venous drainage cannula (Medtronic Inc., Dublin, Ireland) was placed into the left atrium (LA) for right atrial perfusion as well as an 8Fr-angled cannula for LA pressure monitoring. All the remaining branches were ligated. The heart and cannulae were de-aired and connected to the perfusion apparatus (Figure 1C).

End of perfusion

Experimental data were collected for up to 24 h or until end criteria were met. The end criteria were defined as follows: (1) asystole or intractable arrhythmia; (2) LV systolic pressure consistently <30 mmHg for piglet heart experiments and <50% of NEHP baseline for juvenile pig heart experiments; or (3) lactate >7 mmol/L on two consecutive assays separated by 1 h (1). We used all three of these parameters as a benchmark for viability in addition to overall visual appearance of the heart and its contractility. We were required to alter our LV systolic pressure cutoff as we had lower baseline LV pressure readings in the adult heart studies. At the end of 24 h, the hearts were decannulated, drained, weighed, and sent to pathology in formalin.

Tissue analysis

The hearts were weighed immediately after procurement before *ex situ* perfusion and again immediately following the end of perfusion. The weights were compared and data presented as percent weight change. Sections from each cardiac chamber were sampled, weighed (wet weight), and stored in a desiccator for 7 days. These tissue samples were then weighed (dry weight) and the ratio of the wet weight to the dry weight was calculated (wet–dry ratio).

The hearts were sent to pathology for routine hematoxylin and eosin staining. The samples were examined and scored by a veterinary pathologist using a previously described myocardial injury scoring system (1–3). Injury scores ranged from 0 to 3 based on myofiber degeneration, myocardial hemorrhage, interstitial edema, and endothelial changes, with 0 representing no damage and 3 denoting severe. Average scores using the ordinal data for each injury type were reported for each cardiac chamber as well as a combined average for each heart.

Data collection

The primary goal of this study is to evaluate heart function for a period of 24 h using the modified extracorporeal circuit. During each experimental run, hemodynamic parameters including heart rate, aortic flow, pulmonary artery flow, aortic root, and left ventricular and left atrial pressures were continuously monitored and recorded every 30 min. In addition, blood gases, electrolyte panels, and lactate levels were recorded on an hourly basis (Radiometer A/S, Copenhagen NV, Denmark) with electrolyte replacement as needed. Coronary vascular resistance (CVR, mmHg/min/mL) was calculated as a measurement of mean aortic root pressure (mmHg) divided by PA flow (mL/min). Cardiac function was assessed using LV systolic pressures, LA pressures, coronary vascular resistance, and lactate levels. Oxygen content and glucose concentration were measured in the infusion (i.e., AO) and drainage (i.e., PA) blood, and oxygen kinetics (consumption and extraction) were calculated hourly.

CVR was calculated as follows:

$$\mathrm{CVR}\left(\frac{\mathrm{mmHg}}{\mathrm{mL/min}}\right) = \frac{\text{Aortic root mean arterial pressure (mmHg)}}{\text{Pulmonary artery blood flow (mL/min)}}$$

Oxygen consumption was calculated as follows:

$$O_2 \text{ consumption}\left(\frac{mL}{min}\right) = (\text{Arterial } O_2 \text{ Content} - \text{Venous } O_2 \text{ Content}) \times \text{Pulmonary artery blood flow}$$

Statistical analysis

Continuous variables are reported as mean ± standard error. Comparisons between continuous variables were conducted using Student's *t*-test. Heart survival rates were calculated using the Kaplan–Meier method. *p*-values less than 0.05 were considered statistically significant. A multiple comparison test was performed using GraphPad Prism version 10.0.0 for Windows (GraphPad Software, Boston, MA, USA; www.graphpad.com).

Results

An NEHP system was developed for Langendorff perfusion using a blood-derived perfusate (leukocyte/thrombocyte depleted blood). Of the 42 animals, 27 were part of intervention groups including plasma exchange or hemofiltration experiments and 15 were control animals. All 27 hearts in the experimental groups (plasma exchange and hemofiltration) survived up to 24 h. Fourteen (93.3%) control hearts failed between 10 and 21 h and one control heart (6.6%) lasted 24 h (Figure 2).

Pediatric model with plasma exchange

In the plasma exchange experiments, 10 piglet hearts from animals weighing a median 9 kg (8–10 kg) were utilized. Five separate piglet hearts were perfused for 24 h, maintaining physiologic rhythms, contractility, and response to epinephrine challenge, prior to elective termination. An additional five controls were perfused without plasma exchange, none of which were successful through 24 h (failure at 15, 16, 17, 17, and 24 h).

Plasma exchange hearts maintained higher, although not statistically significant, LV systolic pressures at the end of perfusion compared with controls (63 ± 10.9 vs. 37 ± 22.0 mmHg, $p > 0.1$). However, this trend was seen throughout the life of the prep as the plasma exchange hearts were able to recover to 80% of baseline LV systolic pressure compared with 50%–60% of baseline for the control hearts (Figure 3A). Coronary resistance was on average similar for the plasma exchange hearts (1.39 ± 0.36 vs. 01.46 ± 0.79 mmHg/mL/min per 100 g of cardiac tissue, $p > 0.05$). However, in the control group, only one heart lasted 24 h, which increased during the life of the experiment; otherwise, the control group had lower coronary resistance values (Figure 4A). Cardiac metabolism demonstrated significantly higher lactate levels for the control hearts (3.6–7.6 vs. 2.8–4.2 mmol/L), which was statistically significant for most of the experiments (Figure 5A). Increased oxygen metabolism was seen in the plasma exchange hearts (2.89 ± 0.1 vs. 1.8 ± 0.1 mL/min/100 g, $p < 0.05$), compared with the control ones.

The final rate of weight change (from start to end of perfusion) was <2% in the plasma group and >50% in the control group,

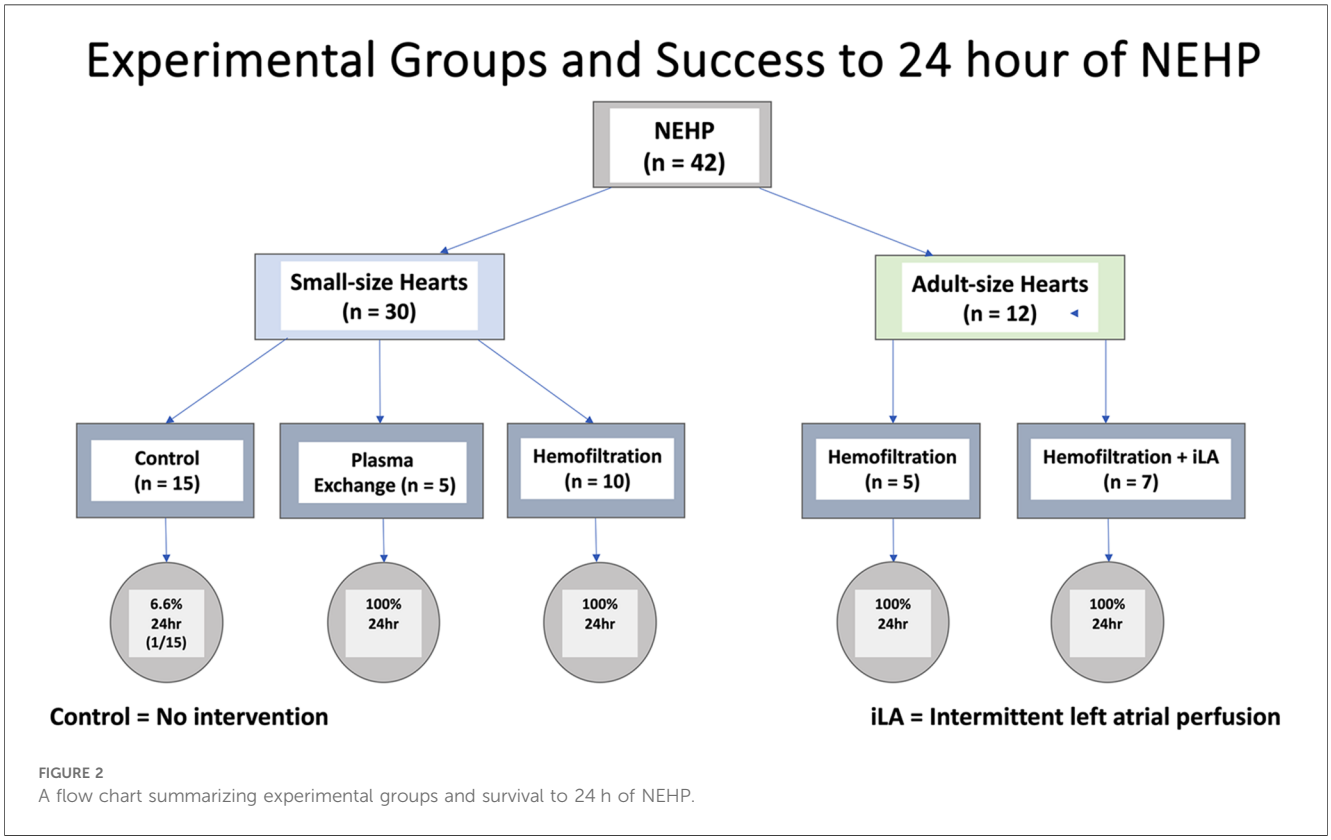

FIGURE 2
A flow chart summarizing experimental groups and survival to 24 h of NEHP.

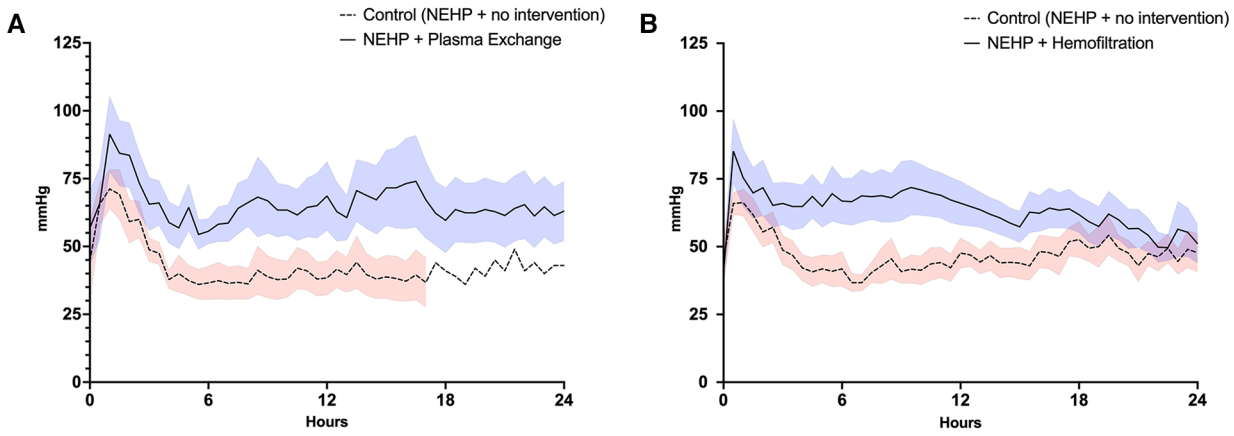

FIGURE 3
Left ventricle systolic pressure during NEHP. (A) Pediatric hearts with plasma exchange; (B) pediatric hearts with hemofiltration.

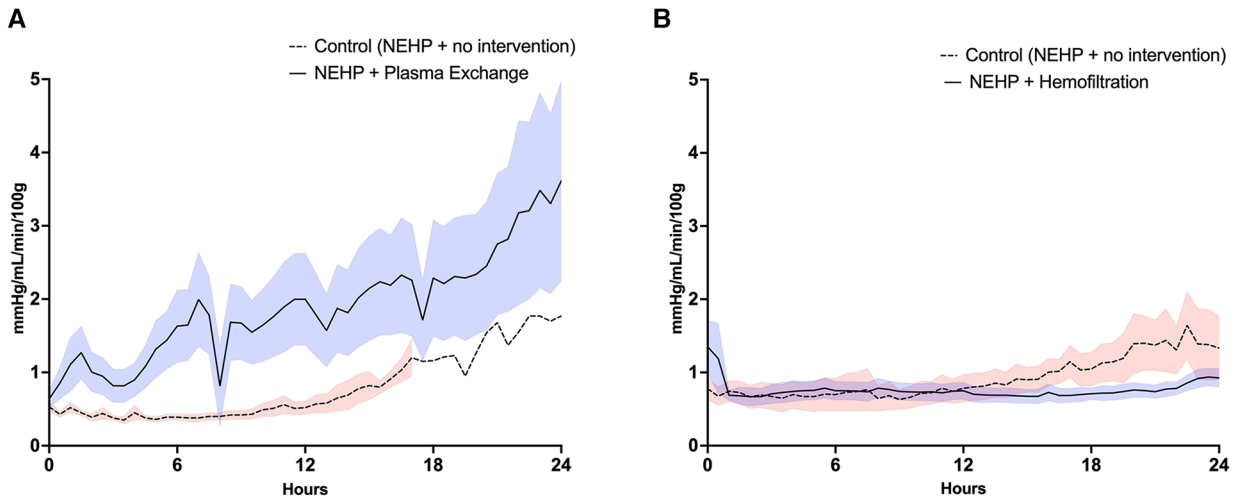

FIGURE 4
Coronary vascular resistance during NEHP. (A) Pediatric hearts with plasma exchange; (B) pediatric hearts with hemofiltration.

$p \leq 0.005$. The wet–dry ratio of the plasma exchange piglet hearts was similar between both groups' LV (5.2 ± 0.2 vs. 5.1 ± 0.2) and RV (5.0 ± 0.5 vs. 4.7 ± 0.3).

Pediatric model with hemofiltration

In the pediatric hemofiltration experiments, there were 28 piglet hearts from animals with an average weight of 8 kg (6–10 kg). Ten hearts received NEHP with hemofiltration and 10 controls were perfused with NEHP alone. Every hemofiltration-treated heart maintained viability at 24 h and the experiment was electively terminated. Only four control hearts were considered viable at 24 h (two failed at 16 h, three at 17 h, and one at 21 h).

LV systolic pressures were significantly higher in the hemofiltration group than in the control group at the 24-h mark (53.5 ± 6.21 vs. 36.3 ± 4.58 mmHg, $p < 0.05$). In addition, the hemofiltration hearts maintained a statistically equivalent LV systolic pressure from start to end of the experiment (71.7 ± 10.30 to 53.5 ± 6.21 mmHg, $p < 0.21$), while the control hearts saw a decreased pressure over the life of the experiment (55.4 ± 5.62 to 36.3 ± 4.58 mmHg, $p < 0.01$) (Figure 3B). Coronary

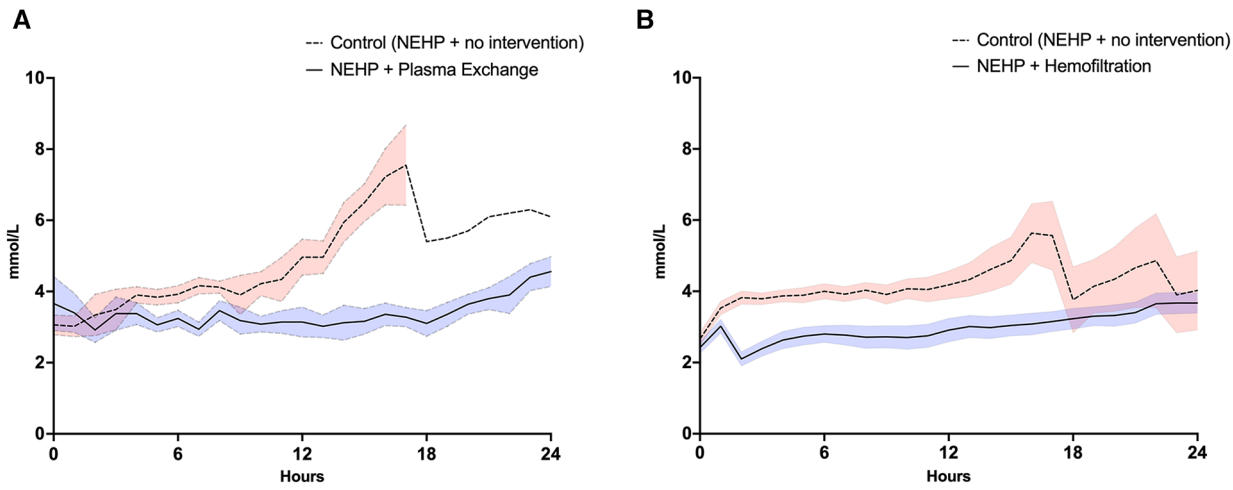

FIGURE 5
Lactate values during NEHP. (**A**) Pediatric hearts with plasma exchange; (**B**) pediatric hearts with hemofiltration.

resistance was maintained from baseline through the end of perfusion in hemofiltration experiments (0.70 ± 0.14 to 0.83 ± 0.11 mmHg/mL/min/100 g of cardiac tissue, $p > 0.05$). In contrast, the coronary resistance doubled from baseline to end of experiment in the control hearts (0.66 ± 0.15 to 1.32 ± 17 mmHg/mL/min/100 g of cardiac tissue, $p < 0.01$) (Figure 4B). At perfusion end, the hemofiltration hearts had a significantly lower coronary resistance than control hearts ($p < 0.05$). Throughout the perfusion period, lactate levels in the hemofiltration hearts were consistently lower in comparison with those in the control group ($p < 0.01$). At the termination of perfusion, lactate levels were measured at 3.67 ± 0.27 mmol/L in the hemofiltration group and 6.20 ± 0.84 mmol/L in the control group ($p < 0.01$) (Figure 5B). Although the control hearts saw a dip in total oxygen consumption from baseline to end of perfusion (0.030 ± 0.005 to 0.018 ± 0.003 mL/min/g tissue, $p = 0.05$), there was no difference between hemofiltration and control hearts.

The final rate of weight change (start to end of perfusion) was <2% in the hemofiltration group and 8%–12% in the control group. The wet–dry ratio of piglet hearts in the hemofiltration group was lower than that of control hearts for both the LV (3.9 ± 0.5 vs. 6.1 ± 0.7, $p = 0.024$) and the RV (4.5 ± 0.6 vs. 5.7 ± 0.4, $p = 0.048$).

An adult-size heart model with hemofiltration

A scientific and translational critique of the piglet (small-size) hearts experiments was the applicability of the results to a larger model. In response, the subsequent two experiments were devised with juvenile pigs to represent an adult model using a second-generation perfusion circuit (Figure 1B). The following data are unpublished and currently under review.

The first experiment successfully perfused five consecutive juvenile pigs, with an average weight of 48.2 ± 3 kg for 24 h. Contractility of the heart was maintained throughout perfusion with a baseline average of 36.6 ± 7.9 mmHg compared with 27 ± 5.5 mmHg at perfusion end (24 h). Similarly, coronary resistance was preserved from baseline 0.79 ± 0.10 mmHg/L/min through the end of perfusion 0.93 ± 0.28 mmHg/L/min. The lactate average at 24 h was 2.6 ± 0.3 mmol/L.

The final rate of weight change (start to end of perfusion) was <2%.

iLA perfusion

The following experiment maintained Langendorff perfusion with intermittent 30-min episodes of left atrial (iLA) perfusion every 4–6 h in seven ($n = 7$) hearts (Figure 1C). Otherwise, the same experimental parameters were maintained.

Mean hemodynamic pressures were continuously monitored with an LV systolic pressure of 18.0 ± 6.3 mmHg, an LA pressure of 5.6 ± 2.6 mmHg, and a calculated coronary vascular resistance of 170 ± 58 mmHg/mL/min. During iLA assessment of the cardiac function, no differences were observed throughout the perfusion process during the 30-min iLA tests at low flows. During iLA, cardiac pressures and waveforms were measured and assessed (Figure 6).

The final rate of weight change (start to end of perfusion) was 8%–10%.

Histopathology and tissue analysis

The pathology scores for each cardiac chamber in the hearts of the control group were higher than those of all experimental

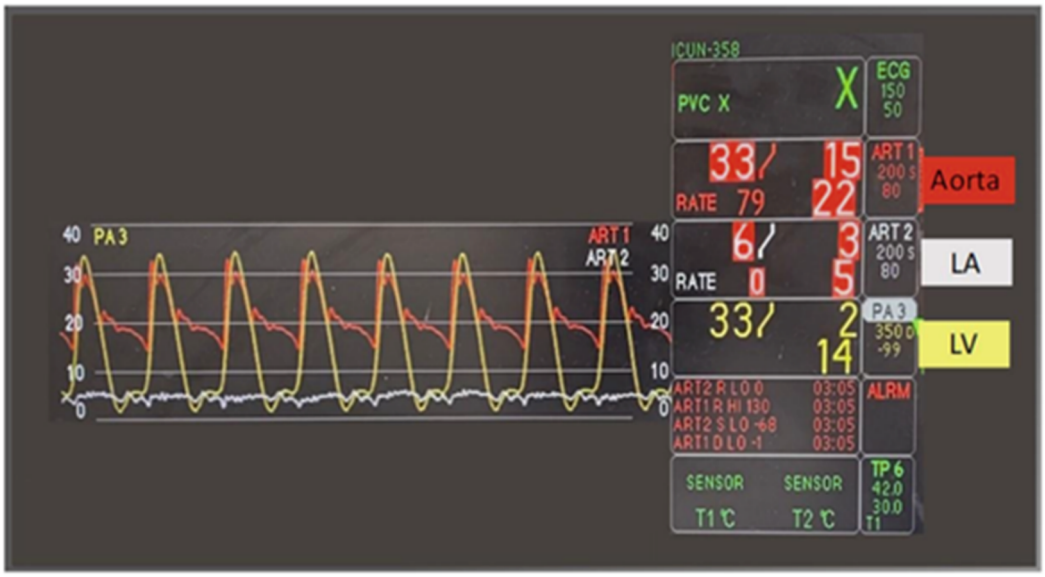

FIGURE 6
Pressure waveform during iLA perfusion low-flow working mode.

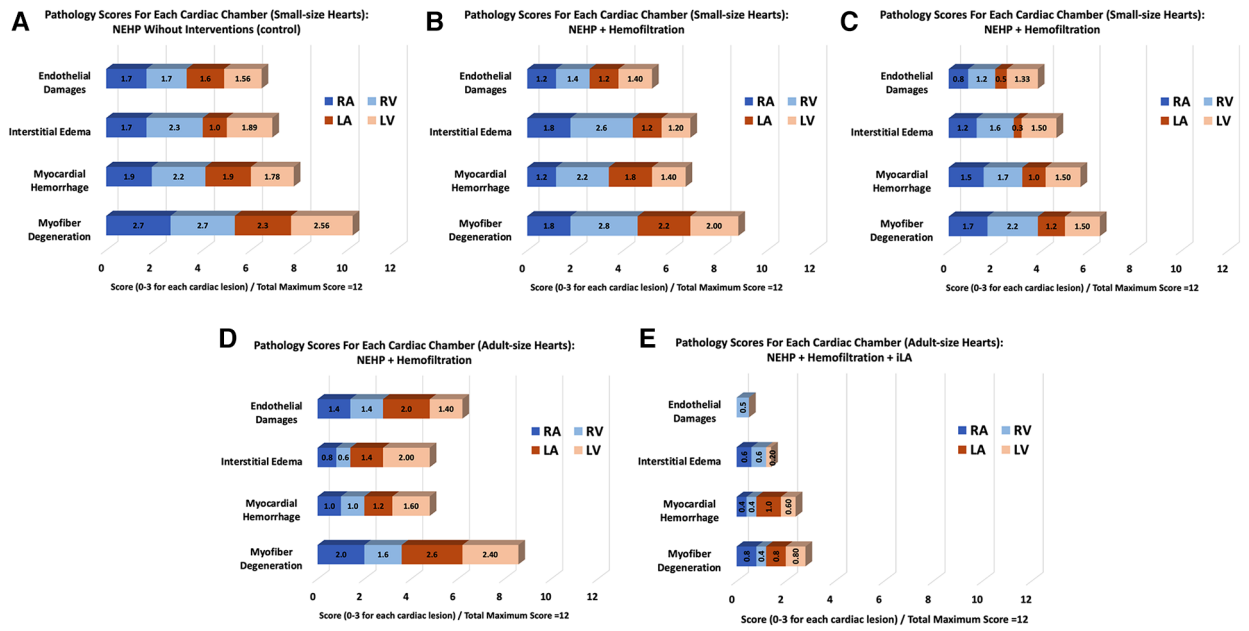

FIGURE 7
A summary of pathological scores for each experimental group. Pediatric (small-size) hearts: (**A**) Control, (**B**) NEHP + plasma exchange,, and (**C**) NEHP + hemofiltration; Adult-size hearts: (**D**) NEHP + hemofiltration and (**E**) NEHP + hemofiltration + iLA.

groups (Figure 7A). All experiments in which the hemofilter was used throughout the NEHP period demonstrated lower interstitial edema and endothelial changes for all chambers of the heart (Figures 7B–E). The hearts in the iLA group had significantly lower scores for all chambers of the heart when compared with those in all other groups (Figure 7E). The scores in the adult-size hearts after NEHP + hemofiltration had a higher value (not significant) compared with the results of the pediatric hearts because of positive contamination of bacteria in the samples. This was due to the fact that biofilm formation was observed in reused plastic components of the NEHP system.

Discussion

This manuscript details experiments performed in our laboratory using NEHP for 24 h, based on our previous successful 3-day perfusion with plasma exchange with paracorporeal animal experiments. The reported experiments were performed in both piglet (small-size) and juvenile pig (adult-size) hearts with varying interventions. Both hemofiltration and plasma exchange groups for piglet hearts performed better than control hearts (no interventions), consistently demonstrating adequate perfusion parameters for the duration of the experiment. The juvenile pig hemofiltered hearts also lasted 24 h and had stable parameters. In our iLA perfusion prep of adult-size hearts, we were able to adequately assess heart function using this model for 24 h and continued to demonstrate adequate heart perfusion and function for the duration of the experiment. These experiments add to the current extended perfusion literature in heart perfusion as well as other organs and demonstrate the feasibility of our circuit and experimental model.

Normothermic *ex situ* heart perfusion has been utilized since 1970, when isolated canine hearts were blood-perfused retrogradely through the aorta in order to evaluate left ventricular performance (4). More recently, studies worked to extend the preservation time with NEHP. Trahanas et al. were able to achieve 12 h of normothermic *ex situ* heart perfusion by perfusing the heart with platelet- and leukocyte-reduced blood supplemented with dextran 40, cell culture media, insulin, and antibiotics with perfusate exchange every 2 h. The successful hearts had lower potassium, lactate, percent weight gain, and pathological injury scores than the hearts that did not achieve 12 h of perfusion (5). Then, McLeod et al. demonstrated that normothermic *ex situ* heart perfusion for up to 72 h using cross-plasma circulation (XC-plasma) from a live, awake paracorporeal sheep was feasible (1). In this study, six ovine hearts were perfused for 72 h using plasma cross-circulation at a rate of 1 L/min with a live, awake paracorporeal sheep. Controls were seven perfused hearts without cross-circulation. Experiments were electively ended at 72 h, and epinephrine (0.1 mg) was delivered to demonstrate hormonal responsiveness. All controls failed at 6–1 h. All six hearts perfused for 72 h maintained normal heart function, metabolism, and responsiveness to epinephrine. Blood gases, electrolytes, and lactate levels were normal and stable throughout the study. From this experience, our group has focused on preserving hearts for 24 h or longer by not only providing warm oxygenated blood, but also filtrating the perfusate and adding nutrition during NEHP.

Clinically, the only commercially available normothermic *ex situ* heart perfusion device is the OCS Heart (Transmedics, Andover, MA, USA). Transplanted hearts that utilized the OCS Heart were shown to have superior 1- and 2-year survival rates, less primary graft failure, less severe acute rejection, and less acute renal failure than transplant hearts preserved with cold Custodiol (6). Patients who received an OCS Heart–perfused heart transplant had comparable 30-day survival rates (7), 2-year survival rates, freedom from cardiac allograft vasculopathy, non-fatal major cardiac events, and biopsy-proven cellular rejection or antibody-mediated rejection to cold storage–preserved hearts (8). In transplants in which a long total ischemic time was expected, patients with OCS Heart–perfused hearts had 30-day and 6-month survival rates of 94.7% and 88%, respectively, and severe LV or RV primary graft dysfunction (PGD) rates of 10.7% (9).

A few cases of successful heart transplantation in humans after extended normothermic perfusion using the OCS system have been reported. The first case was reported by Stamp et al. (10), in which a heart was preserved for 8.5 h with successful transplantation into a 39-year-old recipient. In this case, extracorporeal membrane oxygenation (ECMO) was used immediately after transplant for 17.5 h, and the recipient was extubated after 72 h and discharged after 15 days. The second report with successful human transplantation was published by Kaliyev et al. (11), where hemofiltration was added to the OCS system to preserve a heart for 16 h. The graft was successfully transplanted to a 48 year-old recipient. Similar to the report by Stamp *et al.*, in this second case of prolonged OCS preservation, ECMO was required for 44 h after transplant, and the patient was extubated at 72 h and discharged home after 24 days with normal biventricular function.

These reports corroborate our results from animal data (pig and sheep) and prove the fundamental principle that normothermic perfusion with the addition of a hemofilter can support organs for prolonged periods of time. Further, both groups concluded that despite optimal perfusion parameters and lactate levels, there was PGD and ECMO was required to support recipients immediately after transplant. This is one of our main conclusions, that current biomarkers and parameters used to assess organ viability lack the objectivity to assess organ function as electrolytes and products of cellular metabolism are removed during hemofiltration. Currently, OCS cannot provide direct measurements of donor heart hemodynamic function. Our perfusion apparatus and perfusion protocols allowed for the monitoring of physiological parameters in a working heart, as we discussed in the iLA studies, in a low-flow environment. Other groups have reported the use of pressure–volume loops and surface echocardiography as methods to assess heart function during NEHP (12). iLA also provides a "working" heart mode setting where echocardiography and pressure/volume curves can be used to not only assess the function of hearts but also to monitor the effects of different interventions to improve graft viability during NEHP. In addition, perfusing hearts in working mode has shown some benefits by mitigating oxidative stress, as reported by Dr. Freed's group (13), and myocardial injury, as demonstrated by the pathology in the iLA group. Working mode NEHP has its limitations, as reported by Olkowicz et al. (14). This group reported the relationship between dysregulation of the cardiac metabolome and declining myocardial function during 8 h of NEHP using the STEEN solution^{TM} and red blood cells. They performed physiological measurements by loading the left ventricle to a pressure of 8 mmHg three times during NEHP. In addition, they demonstrated that several metabolic pathways are altered during NEHP, with emphasis on increased inflammatory and oxidative stress response and compromised substrate utilization.

There are lessons from prolonged perfusion of other organs that may impact NEHP. Chapman et al. perfused bovine livers with normothermic blood for 24 h (15). Vogel et al. transplanted porcine livers after perfusing with blood for 48 h. They showed that the livers sustained bile production and metabolic activity for 5 days and there was a 100% survival rate (16). Another focus of *ex situ* liver perfusion studies is the rehabilitation of damaged organs. Schön et al. showed that 4 h of normothermic perfusion was sufficient to recover liver function after an hour of warm ischemia (17). St Peter et al. perfused porcine livers for 24 h after an hour of warm ischemia and the perfused livers demonstrated superior synthetic function, substrate utilization, and perfusion hemodynamics as well as less cellular injury compared with livers preserved with cold storage after warm ischemia (18). This rehabilitation can be applied to heart perfusion if we can further extend *ex situ* heart perfusion to cardiac grafts from marginal donors or from donors after circulatory arrest.

Ex situ lung perfusion has also shown the ability to maintain lung function and rehabilitate damaged lungs. Steen et al. demonstrated that porcine lungs perfused for 6 h and then transplanted and reperfused for 24 h all maintained baseline blood gas transfer and pulmonary vascular resistance (19). Spratt et al. showed that 24 h of *ex situ* lung perfusion improved hemodynamics and compliance after warm ischemia (20). They then transplanted those lungs, reperfused them for 4 h, and found that lung function in the first 8 h of *ex situ* lung perfusion was able to predict lung function posttransplant (21).

Our work adds to the expanding research on normothermic *ex situ* organ perfusion, specifically on heart resuscitation and function assessment. The addition of iLA perfusion enables real-time objective quantifiable cardiac function assessment during NEHP, a unique feature with significant impact during the assessment of marginal donor hearts and hearts from donors after circulatory death.

Limitations

This is a translational model of heart preservation in piglets (small-size hearts) and juvenile pigs (adult-size hearts). The animal model does not simulate the scenario of human heart donation (brain death or cardiopulmonary death physiology) as the animals are young and healthy. In addition, the use of blood products from the blood bank showed negative effects during NEHP, as reported by Chew et al. (22). However, this was a single case report that did not account for the effects of the citrate-based anticoagulant and the age of the blood. Another limitation of our results is related to contamination with bacteria of the histology from the adult-size studies in the NEHP + hemofiltration series. This is due to the fact that some of the NEHP system components were reused during the early studies with juvenile pigs, and we observed a contamination of bacteria and biofilm formation in some plastic components that affected the histological scores.

Conclusion

Prolonged (24 h or more) heart preservation is feasible with our NEHP perfusion technique. LA perfusion, even in coronary flow (working) mode, enables real-time functional assessment during NEHP. To increase the preservation period beyond 24 h, infection control and nutritional support need to be optimized. The current work proves the concept in a large animal model that NEHP has the potential to increase the organ pool by (1) increasing the possible donor/recipient distance; (2) enabling an objective assessment of heart function with the addition of working mode perfusion; (3) considering previously discarded hearts for transplantation; and (4) developing heart-donor-type–specific therapies during NEHP. Further studies that include working mode NEHP, objective parameters to assess heart function, and the development of novel biomarkers to assess heart viability are required to translate prolonged NEHP successfully and routinely into clinical practice.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by The University of Michigan Institutional Animal Care & Use Committee (IACUC). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

BS: Data curation, Formal Analysis, Methodology, Supervision, Writing – original draft, Writing – review & editing. SW: Data curation, Formal Analysis, Methodology, Writing – original draft, Writing – review & editing. CS: Formal Analysis, Writing – original draft, Writing – review & editing. KU: Data curation, Writing – original draft. DP: Formal Analysis, Writing – original draft, Writing – review & editing, Data curation. RB: Writing – original draft, Writing – review & editing, Funding acquisition, Project administration, Resources, Supervision, Visualization. DD: Resources, Supervision, Writing – original draft, Writing – review & editing, Conceptualization, Formal Analysis. AR-P: Conceptualization, Formal Analysis, Resources, Supervision, Writing – original draft, Writing – review & editing, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Visualization.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Lung transplantation following controlled hypothermic storage with a portable lung preservation device: first multicenter European experience

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Introduction: Compared with traditional static ice storage, controlled hypothermic storage (CHS) at 4–10°C may attenuate cold-induced lung injury between procurement and implantation. In this study, we describe the first European lung transplant (LTx) experience with a portable CHS device.

Abbreviations

BMI, body mass index; BREATHE, Laboratory of Respiratory Diseases and Thoracic Surgery; CF, cystic fibrosis; CHS, controlled hypothermic storage; CLAD-BOS, chronic lung allograft dysfunction bronchiolitis obliterans syndrome; COPD, chronic obstructive pulmonary disease; DBD, donation after brain death; DCD, donation after circulatory death; ECMO, extracorporeal membrane oxygenation; FiO2, fraction of inspired oxygen; GUARDIAN-LUNG, Global Utilization And Registry Database for Improved preservAtion of doNor LUNGs; HU, high-urgency; ICU, intensive care unit; ILD, interstitial lung disease; IQR, interquartile range; ISHLT, International Society for Heart and Lung Transplantation; LTx, lung transplantation; NA, not applicable; PaO2, partial oxygen pressure; PGD, primary graft dysfunction; PGD3, primary graft dysfunction grade 3; POD, postoperative day; PVD, pulmonary vascular disease; RCT, randomized controlled trial; SIS, static ice storage; UG, ungradable; VA, venoarterial; VV, venovenous.

Methods: A prospective observational study was conducted of all consecutively performed LTx following CHS (11 November 2022 and 31 January 2024) at two European high-volume centers. The LUNGguard device was used for CHS. The preservation details, total ischemic time, and early postoperative outcomes are described. The data are presented as median (range: minimum–maximum) values.
Results: A total of 36 patients underwent LTx (i.e., 33 bilateral, 2 single LTx, and 1 lobar). The median age was 61 (15–68) years; 58% of the patients were male; 28% of the transplantations had high-urgency status; and 22% were indicated as donation after circulatory death. In 47% of the patients, extracorporeal membrane oxygenation (ECMO) was used for perioperative support. The indications for using the CHS device were overnight bridging ($n = 26$), remote procurement ($n = 4$), rescue allocation ($n = 2$), logistics ($n = 2$), feasibility ($n = 1$), and extended-criteria donor ($n = 1$). The CHS temperature was 6.5°C (3.7°C–9.3°C). The preservation times were 11 h 18 (2 h 42–17 h 9) and 13 h 40 (4 h 5–19 h 36) for the first and second implanted lungs, respectively, whereas the total ischemic times were 13 h 38 (4 h 51–19 h 44) and 15 h 41 (5 h 54–22 h 48), respectively. The primary graft dysfunction grade 3 (PGD3) incidence rates were 33.3% within 72 h and 2.8% at 72 h. Intensive care unit stay was 8 (4–62) days, and the hospital stay was 28 (13–87) days. At the last follow-up [139 (7–446) days], three patients were still hospitalized. One patient died on postoperative day 7 due to ECMO failure. In-hospital Clavien–Dindo complications of 3b were observed in six (17%) patients, and 4a in seven (19%).
Conclusion: CHS seems safe and feasible despite the high-risk recipient and donor profiles, as well as extended preservation times. PGD3 at 72 h was observed in 2.8% of the patients. This technology could postpone LTx to daytime working hours. Larger cohorts and longer-term outcomes are required to confirm these observations.

KEYWORDS

controlled hypothermic storage, lung preservation, overnight bridging, preservation temperature, preservation time, primary graft dysfunction, total ischemic time

1 Introduction

Optimal donor lung preservation is a critical determinant of successful lung transplantation (LTx). For decades, static ice storage (SIS) has been the standard for organ preservation. However, the vulnerability of lung tissue to cold-induced injury caused by near-freezing temperatures may increase the risk of severe primary graft dysfunction (PGD) (1–3). Moreover, preservation on ice is limited to a maximum of 8 h. Therefore, SIS constrains the donor pool and frequently necessitates LTx to be performed overnight under suboptimal working conditions with limited staff. The flaws of SIS have fueled a renewed interest in optimizing donor lung preservation to further improve outcomes after LTx (3–10). Pioneering animal research in the 1990s, followed by the first recent clinical application in Toronto, showed that controlled hypothermic storage (CHS) at 10°C better preserves mitochondrial integrity (3, 4, 6, 8, 11–14). This approach effectively maintains tissue metabolism and mitigates lung injury during the interval between procurement and implantation. Furthermore, CHS enables the prolongation of preservation times, facilitating a shift to planned semi-elective transplant procedures and expanding the donor pool by increasing geographic reach and optimizing donor–recipient matching.

The portable device for CHS (LUNGguard^{TM}) maintains the temperature between 4 and 8°C (15). The system was used for the first time in North America in February 2021 (at Duke University Hospital, North Carolina, USA), and introduced in Europe on 11 November 2022 (with the first European LTx performed using the device at the University Hospitals Leuven, Belgium) (16, 17). No manuscripts on the clinical outcome have been published, to our knowledge. The aim of this study is to provide a descriptive cohort analysis of the first European experience on lung CHS with this device by reporting the perioperative characteristics and short-term outcome.

2 Methods

2.1 Study design

A prospective observational study of all consecutive cases that underwent LTx between 11 November 2022 and 31 January 2024 was conducted at two European high-volume centers: (1) the University Hospitals Leuven (Leuven, Belgium) and (2) the Motol University Hospital (Prague, Czech Republic). The sole inclusion criterion was lung preservation with the CHS device, and there were no exclusion criteria. Data were collected from written and electronic patient files, as well as donor data prospectively

collected by Eurotransplant. The study was approved by the research Ethics Committees of Leuven (S67697) and Prague (EK387/23). Written informed consent was obtained from each patient. The follow-ups were reported until 31 January 2024.

2.2 Controlled hypothermic storage

All lungs were stored in a portable CHS device (LUNGguard) developed by Paragonix Technologies (Waltham, MA, USA). The SherpaCool phase-changing technology enables CHS by maintaining preservation temperatures at 4–8°C for 40 h. The system received the Food and Drug Administration (FDA) clearance in the United States, and the CE (for Conformité Européenne or European Conformity) mark in Europe. A smartphone application connected to a logger and thermometer in the CHS device permits remote continuous real-time monitoring of location and preservation temperature (15).

Routinely, during procurement the lungs were flushed in an antegrade fashion: in Leuven, 4 L OCS^{TM} lung solution (TransMedics, Inc., Andover, MA, USA) was used, whereas in Prague, 6 L Perfadex^{TM} (XVIVO AB, Göteborg, Sweden). After procurement the lungs were split at the donor center and additionally flushed in a retrograde fashion with 0.5–1 L per lung. A maximum of 250 g of ice was used on the bench table, and in Leuven the donor lung surface temperature was controlled by infrared thermal camera prior to storage. Next, the lungs were packed separately in three plastic bags as in our standard approach, and stored in the CHS device: the first bag included the organ itself immersed in 1 L of preservation solution, the second bag was filled with 1 L saline and the first bag, and the third bag contained the first two bags without additional solution. The preservation solution and saline were stored in a fridge at 6°C at the recipient center, and were placed in the CHS device for transport to the donor center, and only removed from the CHS device just prior to its utilization. Finally, after packing, the lungs were stored simultaneously in the CHS device, and the storage temperature was measured continuously through the built-in thermometer of the device.

The clinical protocol for overnight bridging gradually changed with growing experience. At first, lung preservation was only extended with the CHS device for cases with expected cold-flush after 10:00 PM and with recipient anesthesia at 7:30 AM. Eventually, the window of extended preservation was prolonged to cases with expected cold-flush after 6:00 PM.

2.3 Recipient, donor, and procedural variables

The recipient characteristics included the recipient center (Leuven, Prague), sex (male, female), age, body mass index (BMI), indication for LTx [chronic lung allograft dysfunction bronchiolitis obliterans syndrome (CLAD-BOS), chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), interstitial lung disease (ILD), pulmonary vascular disease (PVD)], time on waiting list, high-urgency (HU) listing, duration of preoperative hospitalization, and need for preoperative extracorporeal membrane oxygenation (ECMO).

Donor characteristics were sex (male, female), age, BMI, type of donation [donation after brain death (DBD), donation after circulatory death (DCD)], cause of brain injury (cardiac arrest, cerebral ischemia, intracerebral hemorrhage, status epilepticus, suicide, trauma), intensive care unit (ICU) stay, partial arterial oxygen pressure over the fraction of inspired oxygen [partial oxygen pressure (PaO2)/fraction of inspired oxygen (FiO2)], Oto-score for secretions (0 = none, 1 = minor, 2 = moderate, 3 = major), and Oto-score for chest x-ray findings (0 = clear, 1 = minor, 2 = opacity 1 ≤ lobe, 3 = opacity > 1 lobe) (18).

Lung preservation variables included indication for CHS, preservation temperature, preservation time for the first and second implanted lung, distance between donor and recipient center, and mode of transport. Preservation time was defined as the interval between the moment the lungs were inserted and removed from the CHS device.

The surgical variables were type of LTx (single, bilateral, lobar), surgical approach (anterolateral thoracotomy, clamshell), need and indication for intraoperative ECMO, blood product transfusion (packed cells, fresh frozen plasma, platelets), total ischemic time of the first and second implanted lung, and surgical time. The total ischemic time was defined as the interval between cardiac arrest for DCD, or cross-clamp for DBD, until lung reperfusion in the recipient, hence including both cold and warm ischemic times. Surgical time was defined as the time from the initial incision to final skin closure.

The postoperative outcomes were as follows: need for ECMO, time on the ventilator, extubation status (successful first extubation, reintubation, tracheostomy, death before extubation), PGD at 0/24/48/72 h after LTx, Clavien–Dindo score, ICU stay, and hospital stay. PGD was based on the International Society for Heart and Lung Transplantation (ISHLT) consensus definition and was assessed by pulmonary edema on chest x-ray and PaO2/FiO2 at 0/24/48/72 h post-LTx (19). PGD grade 3 (PGD3) was assigned when the chest x-ray revealed pulmonary edema with a PaO2/FiO2 <200 or when the combination of ECMO with bilateral pulmonary edema on chest x-ray occurred. Data on arterial blood gases were acquired by automated extraction of electronic patient files. The chest x-rays were evaluated retrospectively by two experienced physicians. Postoperative surgical complications were graded according to the Clavien–Dindo classification (20). The longer-term outcomes included follow-up and patient survival.

2.4 Lung transplant procedure and immunosuppression protocol

In Leuven, a routine LTx is performed via bilateral anterolateral thoracotomy in a sequential single LTx fashion, with selective use of ECMO to anticipate and overcome hemodynamic and respiratory instabilities (hypercapnia, PGD, pulmonary hypertension, ventilatory limitations). In Prague, the surgical approach involves a clamshell thoracotomy with sequential single LTx, and protocol use of intraoperative central venoarterial (VA) ECMO. In case of a

single LTx, in both centers a unilateral anterolateral thoracotomy is performed without ECMO.

Immunosuppression consisted of triple therapy with tacrolimus, mycophenolate mofetil, and steroids. The induction immunosuppression used was rabbit antithymocyte globulin (Leuven) or basiliximab (Prague).

2.5 Statistical analysis

Descriptive statistical analyses were performed using Microsoft 365 Excel (Windows). The graphs were plotted with GraphPad Prism10 (San Diego, CA, USA). The continuous variables were summarized as median (range: minimum–maximum) values, and the categorical variables as observed frequencies and percentages.

3 Results

3.1 Recipient and donor characteristics

A total of 160 patients underwent LTx in Leuven ($n = 85$; 53.1%) or Prague ($n = 75$; 46.9%). CHS storage was carried out in 36 LTx cases: 24 (66.7%) in Leuven and 12 (33.3%) in Prague. Most of the patients were men ($n = 21$; 58.3%) aged 61 (15–68) years. The BMI was 26.2 (13.5–29.9) kg/m^2. Indications for LTx were as follows: 17 (47.2%) COPD, 14 (38.9%) ILD, 3 (8.3%) PVD, 1 (2.8%) CF, and 1 (2.8%) CLAD-BOS after LTx. Time on the waiting list was 97.5 (1–826) days. A total of 10 (27.8%) patients were transplanted in a HU setting, following pretransplant hospitalization of 9.5 (2–106) days, with 3 (8.3%) preoperative venovenous (VV) ECMO.

The donor population was predominantly female ($n = 20$; 55.6%), aged 56 (29–94) years, with a BMI of 25.6 (18.0–34.9) kg/m^2. There were 28 DBD (77.8%) and 8 (22.2%) DCD procedures, of which 7 were DCD class 3 (DCD-III) and 1 DCD-IV. The causes of death were varied, with 19 (52.8%) patients dying because of intracerebral hemorrhage, 6 (16.7%) trauma [head injury ($n = 1$) and falling ($n = 5$)], 5 (13.9%) cardiac arrests, 3 (8.3%) cerebral ischemia, 2 (5.6%) suicide [drug intoxication ($n = 1$) and gunshot ($n = 1$)], and 1 (2.8%) status epilepticus. Preoperative ICU stay was 3 (1–12) days. PaO2/FiO2 was 413.5 (264–545). Oto-score for secretions was 1 and chest x-ray findings 0, indicating minor secretions and absence of opacities, respectively. The recipient and donor characteristics are presented in Table 1 and the Supplementary Material.

3.2 Controlled hypothermic storage

Indications for using the CHS device were overnight bridging ($n = 26$; 72.2%), remote procurement ($n = 4$; 11.1%), rescue allocation ($n = 2$; 5.6%), logistics ($n = 2$; 5.6%), feasibility ($n = 1$; 2.8%), and extended-criteria donor ($n = 1$; 2.8%) (advanced donor age: 94 years). Preservation temperature was 6.5°C (3.7°C–9.3°C), and CHS preservation time was 11 h 18 (02 h 42–17 h 09) and 13 h 40 (04 h 05–19 h 36) for the first and second implanted lungs, respectively. The distance between donor and recipient center was 148 (0–980) km, with air transport in 10 (27.8%) cases. The details on lung preservation are summarized in Table 2.

3.3 Surgical variables

Most recipients underwent a full-size bilateral LTx ($n = 33$; 91.7%), while two (5.6%) a single LTx, and one (2.8%) a lobar bilateral LTx (pediatric CF). Bilateral anterolateral thoracotomy was the surgical approach in 25 (69.4%) patients, clamshell in 9 (25%), and a unilateral anterolateral thoracotomy in 2 (5.6%). In 18 (50%) patients intraoperative ECMO was used, of which 1 (2.8%) was due to reperfusion edema of the first implanted lung. Altogether 19 (52.8%) patients required blood products intraoperatively [0.5 (0–20) units]. Total ischemic time was 13 h 38 (04 h 51–19 h 44) and 15 h 41 (05 h 54–22 h 48) for the first and second implanted lungs, respectively. The surgical time was 07 h 00 (02 h 57–13 h 19). The surgical variables are listed in Table 2 and the Supplementary Material.

3.4 Short-term outcomes

Five (13.9%) patients required ECMO postoperatively of which two (5.6%) for suboptimal oxygenation and ventilation caused by lung edema, and three (8.3%) for non-hypoxic reasons. One (2.8%) patient was switched from VV ECMO to VA ECMO due to cardiogenic shock. She died at POD7 after withdrawal of supportive therapy because of irreversible ischemic-hypoxic encephalopathy. Postoperative time on ventilator was 25.5 (6–526) h. Two (5.6%) patients required tracheostomy due to failure from weaning. Within and at 72 h PGD3 was present in 12 (33.3%) patients and one (2.8%) patient, respectively (Figure 1A). ICU stay was 8 (4–62) days, while hospital stay was 28 (13–87) days. During hospitalization, six (17%) patients suffered from a Clavien–Dindo 3b scoring, and seven (19%) from a 4a classification (Figure 1B). Follow-up was 139 (7–446) days. At the final date of follow-up, three (8.3%) patients were still hospitalized, and patient survival was 97.2% ($n = 35$). The postoperative outcomes are outlined in Table 3 and the Supplementary Material.

4 Discussion

This first European experience with the portable LUNGguard shows safe and good short-term outcome for preservation through CHS, and the possibility of converting the transplant procedure to a diurnal activity. The median preservation temperature was 6.5°C. The main indication for using CHS was overnight bridging ($n = 26$; 72.2%). Although maximum

TABLE 1 Recipient and donor characteristics.

	Recipient					Donor				
	Sex (M/F)	Age (years)	Indication for LTx	Time on waiting list (days)	High urgency	Sex (M/F)	Age (years)	Type of donation	ICU stay (days)	PaO2/FiO2
1	M	55	ILD	121	Yes	M	44	DCD-III	12	457
2	F	15	CF	77	No	M	37	DBD	4	374
3	M	66	COPD	515	No	M	35	DCD-III	6	326
4	M	63	ILD	277	Yes	F	63	DBD	2	420
5	F	66	COPD	826	No	F	54	DCD-III	2	486
6	F	53	PVD[a]	500	Yes	F	68	DBD	1	448
7	M	66	ILD	56	No	M	44	DBD	3	361
8	F	64	COPD	651	No	F	59	DCD-III	5	376
9	M	54	ILD	297	No	M	54	DCD-IV	9	264
10	F	64	ILD	235	No	F	74	DBD	4	339
11	F	62	COPD	57	No	F	56	DBD	2	340
12	M	61	COPD	460	No	M	94	DCD-III	8	440
13	F	64	COPD	261	No	F	71	DBD	4	471
14	M	63	COPD	20	No	M	46	DBD	2	321
15	M	22	PVD[ab]	27	No	M	49	DBD	4	463
16	F	59	COPD	507	No	F	75	DBD	3	513
17	F	63	COPD	161	No	F	30	DBD	2	442
18	F	52	ILD	18	No	F	87	DBD	3	486
19	F	61	COPD	168	No	F	70	DBD	2	276
20	M	59	PVD[ac]	2	Yes	M	69	DBD	7	345
21	F	64	COPD	7	No	F	67	DBD	2	396
22	M	48	ILD	6	Yes	M	60	DBD	3	391
23	M	53	ILD	11	Yes	M	68	DBD	1	515
24	M	55	COPD	320	No	M	71	DCD-III	8	421
25	M	56	ILD	227	No	F	56	DBD	4	435
26	M	61	COPD	9	No	M	45	DBD	1	545
27	M	68	ILD	483	No	F	35	DBD	1	407
28	M	49	ILD	1	Yes	F	65	DBD	2	327
29	M	65	COPD	88	No	M	29	DBD	3	377
30	M	47	COPD	107	Yes	M	48	DCD-III	3	427
31	M	68	ILD	26	Yes	F	59	DBD	1	363
32	F	56	ILD	113	No	F	66	DBD	5	465
33	F	60	CLAD-BOS	48	Yes	F	44	DBD	2	390
34	F	65	COPD	72	No	F	36	DBD	7	431
35	M	57	ILD	3	No	F	54	DBD	4	510
36	M	67	COPD	60	No	M	40	DBD	12	337
MV	—	61	—	97.5	—	—	56	—	3	413.5

CF, cystic fibrosis; CLAD-BOS, chronic lung allograft dysfunction bronchiolitis obliterans syndrome; COPD, chronic obstructive pulmonary disease; DBD, donation after brain death; DCD, donation after circulatory death; F, female; FiO2, fraction of inspired oxygen; ICU, intensive care unit; ILD, interstitial lung disease; LTx, lung transplantation; M, male; MV, median value; PaO2, partial oxygen pressure; PVD, pulmonary vascular disease.
[a]Chronic thromboembolic pulmonary hypertension.
[b]Pulmonary capillary hemangiomatosis.
[c]End-stage sarcoidosis with secondary pulmonary hypertension.

preservation (19 h 36) and total ischemic times (22 h 48) importantly exceeded the current limits of 8 and 10 h, respectively, PGD3 incidence at 72 h was 2.8%.

The hypothesis suggesting CHS is associated with improved post-reperfusion outcome compared with SIS was first proposed by the group of Joel Cooper (Toronto) between 1989 and 1993, based on animal research on donor lung preservation temperature (11–14, 21–23). The conclusion of this preclinical research was that the optimal lung preservation temperature was around 8–10°C, allowing the option of extended preservation up to 24 h (11, 21). Three decades later, in 2021, there was a reinstated interest in preservation temperature by Ali et al. and Cypel et al. from the Toronto group, with a first clinical evaluation of five patients receiving LTx after a CHS at 10°C (4). The purpose was overnight bridging and starting the LTx procedure in the morning. Preservation time was 10 h 24 (09 h 55–14 h 48) and 12 h 06 (10 h 54–16 h 30) for the first and second implanted lung, respectively. There was no PGD3 at 72 h, median time on the ventilator was 2 (0–7) days, median hospital stay was 17 (14–26) days, and 30-day survival was 100%. Based on these promising results, the research group led by Cypel set up a prospective non-randomized clinical trial assessing the extension of the static donor lung preservation at 10°C ($n = 70$) vs. SIS ($n = 140$) (9). The lungs were procured and transported in

TABLE 2 Controlled hypothermic storage device and lung transplantation characteristics.

	Controlled hypothermic storage device			Lung transplantation details					
	Indication for CHS device	Preservation temperature (°C)	Preservation time CHS device first/second lung (h)	Type of LTx	Surgical approach	Intraoperative ECMO	Blood products (units)	Total ischemic time first/second lung (h)	Surgical time (h)
1	Feasibility	6.9	04 h 30/06 h 44	BLTx	BAT	No	0	07 h 31/09 h 17	07 h 27
2	Overnight bridging	5.9	07 h 56/11 h 20	Lobar BLTx	BAT	No	4	11 h 26/14 h 29	08 h 25
3	Overnight bridging	5.8	09 h 13/12 h 05	BLTx	BAT	Yes	6	11 h 35/14 h 40	07 h 20
4	Remote procurement	4.0	07 h 46/10 h 06	BLTx	BAT	Yes	20	10 h 42/13 h 12	11 h 01
5	Overnight bridging	6.1	12 h 42/15 h 38	BLTx	BAT	No	3	15 h 53/18 h 55	06 h 36
6	Overnight bridging	6.3	14 h 15/18 h 23	BLTx	BAT	Yes	1	17 h 43/22 h 02	12 h 12
7	Rescue allocation	6.4	06 h 03/NA	Single LTx	UAT	No	0	09 h 11/NA	03 h 23
8	Overnight bridging	8.8	12 h 15/15 h 20	BLTx	BAT	No	2	13 h 58/17 h 18	13 h 19
9	Overnight bridging	9.3	12 h 12/14 h 20	BLTx	BAT	No	0	14 h 59/16 h 51	05 h 39
10	Overnight bridging	5.7	07 h 43/09 h 07	BLTx	BAT	No	0	09 h 41/10 h 59	04 h 43
11	Remote procurement	8.9	04 h 36/06 h 32	BLTx	BAT	No	0	06 h 59/08 h 39	04 h 31
12	Extended-criteria donor[a]	6.9	02 h 42/04 h 20	BLTx	BAT	No	1	05 h 05/06 h 52	05 h 07
13	Overnight bridging	7.0	08 h 40/11 h 58	BLTx	BAT	Yes	3	12 h 03/15 h 23	08 h 35
14	Overnight bridging	7.6	12 h 40/15 h 44	BLTx	BAT	No	0	15 h 41/18 h 53	07 h 10
15	Overnight bridging	6.4	13 h 52/16 h 59	BLTx	BAT	Yes	5	16 h 40/19 h 45	07 h 39
16	Overnight bridging	5.0	14 h 11/16 h 08	BLTx	BAT	No	0	16 h 49/20 h 03	07 h 04
17	Logistics	9.0	04 h 14/05 h 38	BLTx	BAT	No	0	06 h 32/08 h 50	05 h 00
18	Overnight bridging	4.2	15 h 59/17 h 36	BLTx	BAT	No	0	18 h 05/19 h 48	06 h 09
19	Overnight bridging	5.4	13 h 14/16 h 57	BLTx	BAT	No	1	16 h 11/20 h 15	09 h 48
20	Overnight bridging	8.7	11 h 42/16 h 42	BLTx	BAT	No	0	15 h 27/20 h 24	12 h 29
21	Overnight bridging	8.6	13 h 27/15 h 01	BLTx	BAT	Yes	0	15 h 40/17 h 55	05 h 27
22	Overnight bridging	6.6	17 h 09/19 h 36	BLTx	BAT	Yes	14	19 h 44/22 h 48	08 h 21
23	Overnight bridging	6.5	11 h 43/15 h 47	BLTx	BAT	No	6	15 h 05/18 h 59	09 h 01
24	Overnight bridging	5.5	15 h 40/18 h 02	BLTx	BAT	No	0	18 h 03/21 h 16	07 h 23
25	Overnight bridging	8.1	10 h 30/12 h 32	BLTx	Clamshell	Yes	0	12 h 35/14 h 32	06 h 30
26	Overnight bridging	9.0	13 h 35/15 h 50	BLTx	Clamshell	Yes	4	16 h 25/18 h 28	08 h 11
27	Rescue allocation	5.5	10 h 50/NA	Single LTx	UAT	No	0	13 h 32/NA	02 h 57
28	Overnight bridging	6.9	11 h 45/13 h 35	BLTx	Clamshell	Yes	0	14 h 00/15 h 48	07 h 00
29	Remote procurement	3.7	03 h 07/04 h 05	BLTx	Clamshell	Yes	2	04 h 51/05 h 54	05 h 05
30	Overnight bridging	6.4	12 h 19/13 h 55	BLTx	Clamshell	Yes	2	14 h 37/15 h 52	04 h 02
31	Overnight bridging	9.0	10 h 50/12 h 38	BLTx	Clamshell	Yes	0	13 h 19/15 h 04	06 h 40
32	Overnight bridging	5.6	09 h 49/11 h 26	BLTx	Clamshell	No	0	11 h 50/13 h 45	05 h 55
33	Remote procurement	5.9	11 h 45/13 h 45	BLTx	Clamshell	Yes	20	13 h 44/15 h 35	08 h 00
34	Logistics	7.6	10 h 34/12 h 27	BLTx	Clamshell	Yes	0	13 h 14/14 h 16	05 h 20
35	Overnight bridging	6.7	08 h 32/11 h 50	BLTx	BAT	Yes	2	11 h 38/14 h 00	07 h 00
36	Overnight bridging	6.0	10 h 55/12 h 27	BLTx	BAT	Yes	2	12 h 53/14 h 37	05 h 45
MV	—	6.5	11 h 18/13 h 40	—	—	—	0.5	13 h 38/15 h 41	07 h 00

BAT, bilateral anterolateral thoracotomy; BLTx, bilateral lung transplantation; CHS, controlled hypothermic storage; ECMO, extracorporeal membrane oxygenation; LTx, lung transplantation; MV, median value; NA, not applicable; UAT, unilateral anterolateral thoracotomy.
[a]94-year-old male donor.

an ice cooler for 03 h 30 [interquartile range (IQR), 02 h 18–04 h 09], and after arrival in the recipient center, they were preserved in a 10°C temperature-controlled incubator for 07 h 48 (IQR, 05 h 46–09 h 37) until implantation. Preservation time (including lung implantation in this study) was 12 h 28 (IQR, 10 h 14–14 h 12) and 14 h 09 (IQR, 12 h 03–15 h 45) for the first and second implanted lungs, respectively. PGD3 incidence at 72 h was 5.7% vs. 9.3%, and 1-year patient survival was 94% vs. 87%, for the CHS vs. SIS groups, respectively. Minor differences were observed in ICU stay (5 vs. 5 days), hospital stay (25 vs. 30 days), and need for postoperative ECMO (5.7% vs. 9.3%).

In February 2022, the commercially portable CHS device LUNGguard was first implemented in North America. Specifically for this device, the clinical non-randomized post-market registry study "Global Utilization And Registry Database for Improved preservAtion of doNor LUNGs' (GUARDIAN-LUNG) (NCT04930289) was started, with the objective of comparing the outcomes after LTx by CHS vs. SIS (24, 25). Preliminary registry data about the North American experience was presented at ISHLT 2023, enrolling 86 LUNGguard and 90 SIS patients. The median preservation temperature was 4.9°C, total ischemic time was 7 h 26 ± 1 h 51, and PGD3 incidence at 72 h was 8.1% (7/86).

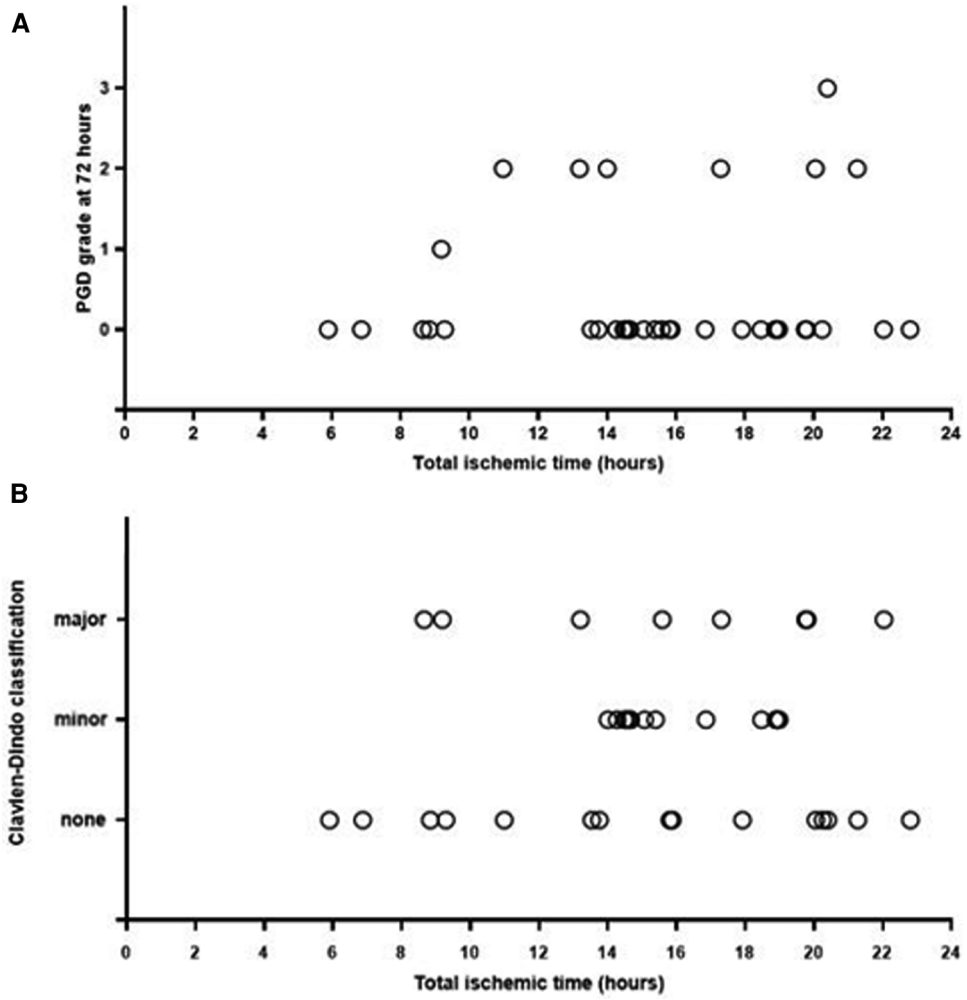

FIGURE 1
(A) Longest total ischemic time (h) and PGD grade (0–1–2–3) at 72 h. (B) Longest total ischemic time (hours) and Clavien–Dindo classification (none: grade 1–2, minor: grade 3a–3b, major: grade 4a–4b–5).

The CHS cohort had a clinically relevant 54% reduction in PGD3 incidence at 72 h ($p = 0.058$) compared with SIS, and was also associated with significantly improved 1-year estimated patient survival [CHS 92.7% vs. SIS 82.2% ($p = 0.02$)] (26).

Our manuscript describes the implementation of this CHS device over a 14-month period in two European centers. It is worth mentioning that the preservation temperature in our cohort was higher compared with the GUARDIAN-LUNG (6.5°C vs. 4.9°C). Both Leuven and Prague attempt to reach higher temperatures, based on the favorable outcome of lungs preserved at 10°C (4, 8, 9). Therefore, we adopted a strategy in which we use maximum 250 g of ice on the bench table, and target a donor lung surface temperature between 8°C and 12°C prior to storage in the CHS device. We observed that the starting surface temperature of the donor lung directs the average preservation temperature afterward: starting temperatures >10°C vs. 8–10°C vs. <8°C were associated with average preservation temperatures above 8°C vs. 6–8°C vs. 4–6°C, respectively. The duration of preservation also influences the temperature curve, which changed during storage, finding equilibrium around 6°C.

Another major difference from the GUARDIAN-LUNG analysis concerns total ischemic time. Compared with the registry with total ischemic time of 7 h 26 ± 1 h 51, we report considerably longer total ischemic times with 15 h 41 (05 h 54–22 h 48) for the second implanted lung. In 52.8% ($n = 19$) of the patients, total ischemic times exceeded 15 h, with a maximum of 22 h 48. Nevertheless, PGD3 at 72 h was only 2.8% in our series. The promising findings of this first European experience with extended preservation and total ischemic times have encouraged Leuven and Prague to implement and standardize overnight bridging ($n = 26$; 72.2%) allowing a shift toward transplantation during the daytime. The literature suggests that nocturnal transplantation might be associated with worse outcomes because of limited resources, shortage of personnel, and lesser technical expertise (27–32). Moreover, fatigue and sleep deprivation of the LTx team might have a negative repercussion on the cognitive

TABLE 3 Postoperative outcomes.

	Postoperative ECMO	Time on ventilator (h)	PGD at 0/24/48/72 h	ICU stay (days)	Hospital stay (days)	Clavien–Dindo score
1	No	37	0/0/0/0	5	19	2
2	No	44	3/2/0/0	14	26	3a
3	No	42	0/0/0/0	6	31	3a
4	No	NA	3/3/3/2	62	87	4a
5	No	48[a]	0/0/0/0	9	30	3b
6	Yes	108	UG/UG/UG/0	16	32	4a
7	No	16	0/0/2/1	4	18	4a
8	Yes	NA	3/3/3/2	7[b]	7[b]	5
9	No	29	3/2/0/0	8	16	3b
10	No	42	0/2/2/2	7	21	2
11	No	19	1/0/0/0	7	83	4a
12	No	15	0/0/0/0	4	31	2
13	Yes	86	3/3/3/0	15	45	3a
14	No	22	0/0/1/0	5	30	3a
15	Yes	64	UG/UG/UG/0	14	34	4a
16	No	20	3/2/2/2	8	28	2
17	No	19	0/0/0/0	7	40	2
18	No	49	0/0/3/0	12	27	4a
19	No	57	3/0/1/0	9	24	2
20	No	17	2/2/3/3	5	20	2
21	No	18	0/0/0/0	7	19	2
22	Yes	39	2/3/2/0	8	Ongoing	2
23	No	57	1/3/0/0	Ongoing	Ongoing	3b
24	No	19	2/1/3/2	Ongoing	Ongoing	2
25	No	6	0/0/0/0	11	20	3a
26	No	7	0/0/0/0	11	34	3b
27	No	10	0/0/0/0	7	13	2
28	No	17	0/0/0/0	7	20	2
29	No	31	0/0/0/0	11	19	2
30	No	21	0/0/0/0	8	19	2
31	No	41	0/0/0/0	46	52	3b
32	No	8	0/0/0/0	7	20	2
33	No	526[a]	0/0/2/0	44	51	4a
34	No	12	0/0/0/0	9	34	3a
35	No	47	2/1/1/2	14	26	3a
36	No	6	0/0/0/0	20	32	3b
MV	—	25.5	—	8	28	—

ECMO, extracorporeal membrane oxygenation; ICU, intensive care unit; PGD, primary graft dysfunction; MV, median value; UG, ungradable.
[a]Tracheostomy due to failure from weaning.
[b]Death on day 7 postoperatively.

and psychomotor skills. Accordingly, the LTx policies of Leuven and Prague have considerably changed, with focus on flexibility and overnight bridging when donor cross-clamp time is planned after 6:00 PM. After procurement, the lungs are stored in the CHS device unattended in the surgical theater of the recipient center. Patient induction occurs the next day at 7:30 AM, and the LTx is performed during the daytime in optimal conditions with a well-rested team and maximal medical expertise. In addition, extended preservation and ischemic times allow expansion of the donor pool through long-distance lung procurement, rescue allocation, facilitation of immunological crossmatch test, and acceptance of a second pair of donor lungs in case of simultaneous or overlapping LTx.

Last but not least, we reported a higher number of DCD procedures ($n = 8$; 22.2%) compared with the registry [CHS $n =$ 15/85 (17.6%) and SIS $n = 6/90$ (6.7%)] (26). These findings can be attributed to the rapidly growing experiences of Leuven with DCD procedures (Leuven $n = 7$ vs. Prague $n = 1$). In fact, based on the favorable long-term survival in DCD-III and DBD lung donor recipients, as reported by ISHLT in 2019, Leuven increasingly performs DCD procurements to expand the donor pool (33).

Several questions on CHS remain, concerning the ideal temperature, extended vs. short preservation, long-term outcome, and potential benefits for extended-criteria donors. Randomized controlled trials (RCTs) and a propensity-matched study from a large GUARDIAN-LUNG cohort ($n = 500$) are awaited (24, 25). Furthermore, a multicenter RCT is currently being conducted by Toronto (X°Port Lung Transport Device, Traferox Technologies Inc.): "Safety of 10°C Lung Preservation Versus Standard of Care: A Multi-Center Prospective Non-Inferiority Trial" (NCT05898776), comparing 160 CHS vs. 160 SIS (24, 34) cases.

5 Conclusion

CHS by LUNGguard seems feasible and safe, despite the relatively high-risk recipient and donor profiles (DCD 22.2%) and extended preservation periods. PGD3 at 72 h of 2.8% was observed in this series. The CHS technology potentially allows overnight bridging and shifting toward daytime transplantation, optimizing working conditions. However, several questions remain and multicenter randomized trials are awaited.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary Material**; further inquiries can be directed to the corresponding author.

Ethics statement

The study was approved by the research Ethics Committees of Leuven (S67697) and Prague (EK387/23). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) and minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

A-LP: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. RN: Writing – original draft, Writing – review & editing, Conceptualization, Investigation, Methodology, Software, Data curation. DVR: Writing – review & editing, Conceptualization. JVS: Writing – review & editing, Conceptualization, Data curation, Investigation, Methodology, Software. EP: Writing – review & editing. CMV: Writing – review & editing. AB: Writing – review & editing. XJ: Writing – review & editing. KD: Writing – review & editing, Data curation, Software. PDL: Writing – review & editing. HVV: Writing – review & editing. LD: Writing – review & editing. YJ: Writing – review & editing. JaP: Writing – review & editing. AN: Writing – review & editing. SB: Writing – review & editing. CI: Writing – review & editing. BJ: Writing – review & editing. LG: Writing – review & editing. LDS: Writing – review & editing. RV: Writing – review & editing. MS: Writing – review & editing. JaV: Writing – review & editing. JK: Writing – review & editing. JT: Writing – review & editing. JH: Writing – review & editing. ZOS: Writing – review & editing. JiP: Writing – review & editing. JS: Writing – review & editing. JiV: Writing – review & editing. RL: Writing – review & editing. LJC: Writing – original draft, Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Software, Supervision, Writing – review & editing.

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Conflict of interest

In relation to this manuscript, we disclose that Paragonix granted five LUNGguard^{TM} devices to the authors to test their feasibility. No financial compensation was granted.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2024.1370543/full#supplementary-material

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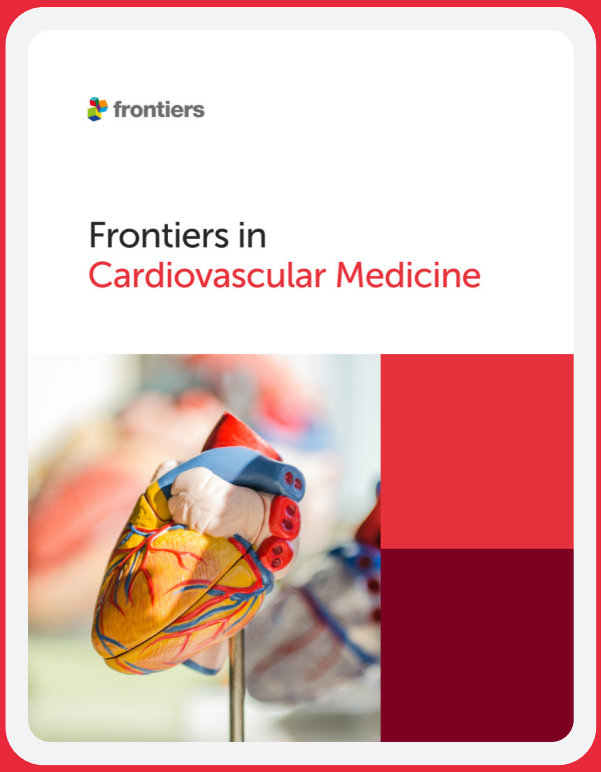

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