Transport-Associated Vibrational Stress Triggers Drug-Reversible Apoptosis and Cardiac Allograft Failure in Mice

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ABSTRACT Increasingly complex and long-range donor organ allocation routes coupled with implementation of unmanned aerial vehicles (UAVs) have prompted investigations of the conditions affecting organs once packaged for shipment. Our group has previously demonstrated that different modes of organ transport exert unique environmental stressors, in particular vibration. Using a mouse heart transplant model, we demonstrated that vibrational forces exert tangible, cellular effects in the form of cardiomyocyte apoptosis and cytoskeletal derangement. Functionally, these changes translated into accelerated allograft loss. Notably, administration of an apoptosis inhibitor, Z-VAD-FMK, helped to ameliorate the detrimental cellular and functional effects of mechanical vibration in a dose-dependent manner. These findings constitute one of the first reports of the negative impact of transit environment on transplant outcomes, a contributing mechanism underpinning this effect, and a potential agent to prophylax against this process. Given current limitations in measuring donor organ transit environments *in situ*, further study is required to better characterize the impact of transport environment and to potentially improve the care of donor organs during shipment. **Clinical and Translational Impact Statement:** We show that apoptosis inhibitor, Z-VAD-FMK, ameliorated transport-related vibrational stress in murine heart transplants, which presents a potential therapeutic or preservation solution additive for future use in transporting donor organs.

INDEX TERMS Organ transplantation, allograft failure, transportation innovation, vibration, transport-related stress.

I. INTRODUCTION

Donor organ allocation strategies have continued to evolve in an effort to improve access equality for this precious resource, resulting in matched organs having to travel greater distances to recipients [1]. These organs accrue additional cold ischemia time (CIT) as their travel distance increases, contributing to increased rates of delayed graft function (DGF), increased recipient length of stay, and an overall decline in transplant outcomes [2], [3]. Given the evolving nature of organ allocation systems and ever-expanding match radii to improve organ sharing in the United States,

understanding the full impact of transport-related stressors on donor tissues is critical.

Our group's efforts to innovate donor organ shipment through the use of unmanned aerial vehicles (UAVs) prompted an investigation of the donor organ's in-flight environment [4]. Moving time-sensitive human organs directly from donor hospital to recipient hospital via UAV could simplify the current, complex process that requires multiple nodes of contact, which subsequently increases CIT. Given that UAV transported organs would be completely unattended, we reasoned that tracking and monitoring of the

organ in transit would be important both for logistics as well as for ensuring organ quality and safety.

At this time, there are no standard of care systems for monitoring donor organs in static cold storage once they have been sterilely packaged for shipment. We subsequently developed a sterile sensor array to monitor a shipped organ's biophysiological condition and GPS location [4], [5]. Comparing the shipment environment of organs shipped by UAV to fixed-wing aircraft or ground transport, we found large variations in vibration and pressure based on the mode of transportation. UAV transport was associated with smaller changes in vibrational stress (<0.5G) compared to fixed-wing transport (>2.0G), although there were no differences between UAV flight and ground-based transport [4], [5]. Furthermore, organs transported via UAV experience pressure fluctuations (ΔP 0.37-0.86 kPa), which are obviated in the pressure-controlled cabins of fixed-wing aircraft. Taken together, these findings suggest that explanted donor organs experience a variety of environmental forces during shipment, warranting investigation of their impact on transplant outcomes.

While there is limited published data on the impact of environmental forces on donor organ quality for transplant, transit-related stressors (temperature, vibration, pressure) have been shown to negatively impact *ex vivo* tissues. Temperature variance above or below the optimal 4-8°C used for static cold storage causes increased hypoxia-induced cell death or ice crystal-mediated destruction of tissue architecture [6], [7]. Vibrational forces cause sheer stress at the cellular and tissue level, resulting in red blood cell destruction, mechanotransduction-mediated oxidative injury in pulmonary endothelium, endothelial cytoskeleton architectural changes, and disruption of fibroblast adhesion [8], [9], [10], [11], [12]. In terms of inflammatory responses, rat spinal cord exposed to a tissue-specific resonant vibrational frequency triggered increased levels of IL-2, IL-6, TNFα, VEGF, IL-10, and IL-4 compared to non-resonant frequencies [13]. Hypobaric conditions, encountered in both fixed-wing and UAV transport, have also been shown to negatively impact tissue homeostasis. Low pressures result in increased hemolysis in blood samples [14], decreased cerebral perfusion and mean arterial pressures in swine [15], and exacerbation of neuronal injury in rats with subsequent motor impairment [16]. Furthermore, donor livers transported by fixed-wing aircraft experienced worse one-year allograft survival and increased rates of primary nonfunction compared to those shipped via ground transport [17].

Reasoning that CIT-associated risks extend beyond time alone, we sought to establish the significance of individual forces that may affect an organ during shipment. Given the susceptibility of *ex vivo* tissues to damage via environmental stressors, our group hypothesized that the shipment environment, and in particular vibrational forces, may also negatively impact donor organ transplant outcomes. We subsequently developed a murine model of heterotopic cardiac transplantation in which donor allografts were exposed to vibrational stress prior to implantation, thought

to be proportional to that experienced during air transport. We subsequently analyzed changes in donor myocardial cell viability and tissue architecture as well as allograft longevity.

II. METHODS

A. MICE
Wild type C57BL/6J (H-2^b) and BALB/cJ (H-2^d) female mice (6-8 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific pathogen-free conditions. All animal experiments and methods were performed in accordance with the relevant guidelines and regulations set by the Office of Animal Welfare Assurance under the approval of the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine (protocol number 0620015), approved August 11, 2020.

B. HETEROTOPIC CARDIAC TRANSPLANTATION
Fully vascularized C57BL/6J hearts were heterotopically transplanted by anastomosing the aorta and pulmonary artery of the allograft in an end-to-side fashion to the aorta and vena cava, respectively, of BALB/cJ recipients under anesthesia, as previously described [18]. All recipients were administered 250μg of anti-CD40L monoclonal antibody (BioXcell, Lebanon, NH) via intraperitoneal (i.p.) injection on the day of transplantation. Graft function was monitored every other day for the first week and then twice weekly by abdominal palpitation. Failure was defined as complete cessation of a palpable beat.

C. REAGENTS
Z-VAD-FMK (Sigma, St Louis, MO), a pan-caspase inhibitor, was reconstituted in DMSO and administered to donor and/or recipient mice at a single dose of 1 mg/kg body weight i.p. 30 minutes prior to explant of donor hearts (Z-VAD1) or an additional dose of Z-VAD-FMK was given to recipients following completion of transplant (Z-VAD2). DMSO alone was used as a control. Z-VAD-FMK was administered to cell culture at a concentration of 1, 5, 10, or 25uM.

D. HISTOPATHOLOGY
Explanted donor hearts were exposed to mechanical vibration, subsequently fixed in 10% formalin solution (HT5011, Sigma), and then embedded in paraffin. Tissue blocks were subsequently sectioned (5μm), processed, and stained with H&E.

E. IMMUNOHISTOCHEMISTRY
Explanted donor hearts were exposed to mechanical vibration, subsequently embedded in OCT compound (Sakura Finetek USA, Torrance, CA), and preserved at -80°C. Cryo sections (5μm) were fixed in cold acetone:methanol (1:1). After blocking with 5% normal goat serum (BioXcell), sections were stained with anti-Annexin V (Proteintech, Rosemount, IL) or anti-ACTC1 (LS Bio, Seattle, WA) primary antibodies overnight at 4°C. Sections were then stained with Alexa Fluor 488- or 647-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA),

respectively, for 1 hour at 4 °C. This was followed by fixation with 4% paraformaldehyde (Alfa Aesar, Tewksbury, MA). Sections were then mounted with Prolong Gold Antifade with DAPI (Invitrogen, Carlsbad, CA), coverslipped, and imaged using a LSM 510 fluorescent microscope (Zeiss, Oberkochen, Germany).

F. MECHANICAL VIBRATION

A horizontally circulating Orbi-Shaker (Benchmark Scientific, Sayreville, NJ) was used to mechanically vibrate explanted donor hearts and H9C2 cells. Hearts were submerged in PBS in 15 ml falcon tubes, which were secured in ice-containing cooling boxes. H9C2 cells were seeded on 6-well plates at a density of 1×10^6 cells/1 mL/well in DMEM medium (Gibco, Waltham, MA) and cultured overnight. Mechanical vibration was applied at a speed of 250 rpm in horizontal waves to grafts and cell culture plates, placed on the center of non-slip, rubber-coated platform and secured with taping. After 10 minutes of vibration, donor grafts were implanted into recipients and cell culture plates were cultured without vibration for 48h. Control donor hearts and cell culture plates were treated identically, but not exposed to vibration.

G. CELL VIABILITY AND CELL PROLIFERATION

H9C2 cells, a rat cardiovascular myoblast cell line, were kindly gifted from Dr. Dong-ik Lee (Johns Hopkins University, Baltimore, MD) and cultured in DMEM medium (Gibco) supplemented with 10% FBS and 1x Penicillin/Streptomycin (Gemini, West Sacramento, CA) at 37°C. For viability analysis, H9C2 myoblast cells were exposed to vibratory stimulation, incubated for 48h, and then washed with 1x PBS, detached by trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA) (Gibco), and mixed with 0.4% trypan blue solution (Sigma-Aldrich, St Louis, MO). Stained cells were counted using a hemocytometer and inverted microscope. Cell viability represented as proportion of unstained cells out of total cells. H9C2 cells were incubated overnight with or without Z-VAD-FMK and exposed to vibratory stimulation of 250 rpm for 10 minutes the following day using the same shaker as allograft experiments.

H. STATISTICAL ANALYSIS

Data presented as mean ± standard error of mean (SEM) or mean ± standard deviation (SD). Student's *t*-test was used to compare 2 treatment groups and one-way ANOVA test was used to compare multiple groups. Kaplan-Meier survival curves were analyzed using Logrank (mantel-cox) test. A *p* value of <0.05 was considered statistically significant. Data analyzed using Graph Pad Prism V6 (LA Jolla, CA, USA).

III. RESULTS

A. VIBRATION LEADS TO ACCELERATED CARDIAC ALLOGRAFT FAILURE

We first examined the effect of vibration on the survival of allogeneic heart transplants. Given that aircraft can
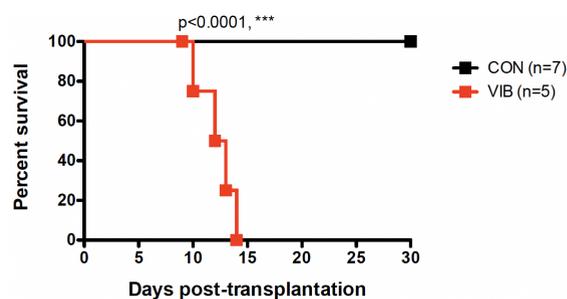

FIGURE 1. Kaplan-Meier survival curve of cardiac allografts (C57BL/6J to BALB/cJ) with and without mechanical vibration. Explanted donor hearts were exposed to mechanical vibration (red) prior to transplant and subsequent anti-CD40L co-stimulation blockade. Donor hearts transplanted into control recipients (black) did not receive mechanical vibration. Median survival times (MSTs) were compared using Logrank (mantel-cox) test. p = 0.0003.

generate vibration ranging from 5 Hz (helicopters) [19] up to 40 Hz (fixed wing aircraft) [20] over transport times that can span hours, we modelled the minimum vibration stimulation of this range using a conversion of 4 Hz to 250 rpm (1 Hz = 60 rpm) over 10 minutes (e.g. explanted C57BL/6J donor hearts were exposed to mechanical vibration at a rate of 250 rpm for 10 minutes). Next, explanted hearts were immediately transplanted into fully MHC mismatched recipient BALB/cJ mice. Given rapid rejection of mismatched treatment and control hearts without immunosuppression within 7 days (data not shown), both control and vibrated groups received anti-CD40L co-stimulatory blockade to allow for more careful comparison of changes in transplant outcomes over time [20], [21]. Vibration significantly shortened cardiac allograft survival (vibration group median survival time (MST) 13 days, n=5; control MST 40 days, n=7; p=0.0003) when compared with non-vibrated control donor hearts (**Fig. 1**). None of the transplanted hearts developed primary non function, or failure within 72 hours.

B. VIBRATED CARDIAC TISSUE DISPLAYS INCREASED APOPTOSIS

To further investigate the cellular level effect of vibration, donor allografts were analyzed by microscopy. Histologic analysis of explanted donor hearts immediately following mechanical vibration, without transplantation, demonstrated a derangement of cellular structures, increased cardiomyocyte edema, and dissolved myofibers (**Fig. 2A, 2B, & Suppl. Fig. 1**). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was then applied to donor tissue to assess for vibration-induced cardiomyocyte apoptosis. In vibration-exposed hearts, there was a marked increase in TUNEL positive cardiomyocytes (**Fig. 2C & D**), suggesting an increase in apoptosis after mechanical vibration. To further assess vibration-induced cell death pathways, the same histologic samples were stained for Annexin V. Similarly, vibrated donor heart tissue displayed increased Annexin V expression in both epicardium and myocardium (**Fig. 3**).
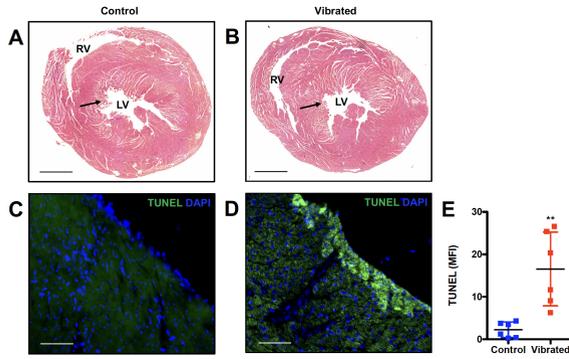
FIGURE 2. Representative H&E staining of transverse cardiac tissue sections (4X magnification, scale bar: 1mm) from explanted (A) non-vibrated donor heart group and (B) vibrated donor heart group. Representative TUNEL staining (200X magnification, scale bar: 125 μm) of transverse myocardial tissue sections, positive cells labelled in green. Minimal TUNEL-positive cells in non-vibrated control heart (C) compared to strong signal in vibrated heart (D). MFI quantitation of TUNEL stain presented as mean ± SEM (E). H&E, hematoxylin-eosin; MFI, mean fluorescence intensity; SEM, standard error of mean; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling. Student's t-test used to compare treatment groups. **p < 0.01.
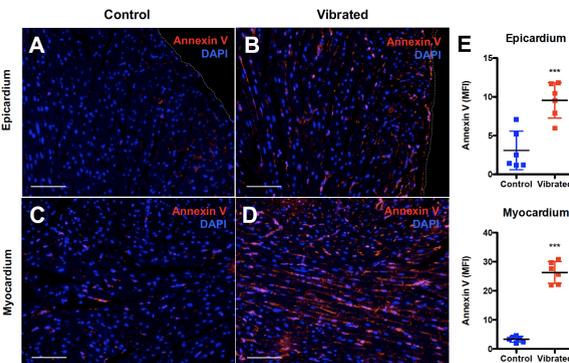

FIGURE 3. Representative immunohistochemistry images of transverse cardiac tissue sections (200X magnification, scale bar: 125 μm) from non-vibrated control heart (A and C) and vibrated heart (B and D) using anti-annexin V (red) and DAPI (blue). MFI quantitation of Annexin V stain of epicardium and myocardium presented as mean ± SEM (E). Student's t-test used to compare treatment groups. ***p < 0.001.

C. VIBRATED DONOR HEARTS SHOWED DERANGED CYTOSKELETAL STRUCTURE

To investigate the effects of mechanical vibration on cardiac cytoskeletal structures, cardiac muscle alpha actin (ACTC1) was identified by immunofluorescence analysis. While control hearts showed well-organized and distributed actin cytoskeletal bands, vibration-exposed hearts exhibited disarray of actin filaments, suggesting abnormal myocardial function (**Fig. 4**).

D. ANTI-APOPTOTIC TREATMENT PROTECTED AGAINST VIBRATION-INDUCED GRAFT FAILURE

To further test the relationship between vibration-induced apoptosis and accelerated allograft failure, donor hearts were pre-treated with a chemical inhibitor of apoptosis prior to mechanical vibration and transplant. Donor mice were given 1 dose of Z-VAD-FMK, an irreversible broad-spectrum caspase inhibitor, 30 minutes prior to procurement of donor
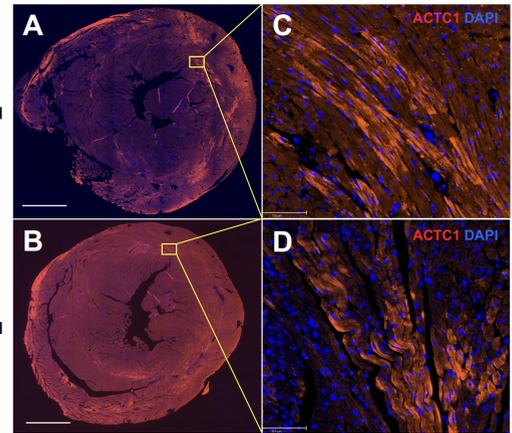

FIGURE 4. Representative immunohistochemistry images of transverse cardiac tissue sections from non-vibrated control heart (A and C) and vibrated heart (B and D) using anti-ACTC1 (red). Magnification: 4X, scale bar: 1mm (A and B) and 200X, scale bar: 125 μm (C and D).
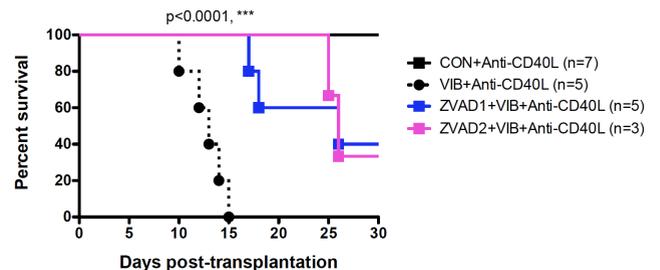

FIGURE 5. Kaplan-Meier survival curves of cardiac allografts (C57BL/6J to BALB/cJ) with and without Z-VAD-FMK treatment. Donor mice were administered Z-VAD-FMK 30 minutes prior to donor heart procurement and vibration (ZVAD1; blue) or one dose of Z-VAD-FMK administered to donors pre-procurement and vibration while a second dose was given to recipients at the time of surgical closure (ZVAD2; pink) with concomitant anti-CD40L treatment. Non-Z-VAD-FMK controls represent vibrated donor hearts with concomitant anti-CD40L treatment (black circle), and non-vibrated donor hearts (black square), as depicted in Figure 1. MSTs were compared using Logrank (mantel-cox) test. p < 0.0001 comparing ZVAD1 or ZVAD2 to vibrated control.

hearts (ZVAD1). In a separate cohort, 1 dose of Z-VAD-FMK was administered to donors pre-procurement while a second dose was given to recipients at the time of surgical closure (ZVAD2). Treatment with either regimen of Z-VAD-FMK significantly prolonged allograft survival compared to control recipients receiving vibrated hearts without anti-apoptotic agent (ZVAD1 MST 26 days, n = 5; ZVAD2 MST 26 days, n = 3; p < 0.0001 comparing ZVAD1 or ZVAD2 to vibrated control). However, there was no difference in allograft outcomes between ZVAD1 and ZVAD2 cohorts, showing that an additional dose of apoptosis inhibitor given to recipients did not provide additional allograft protection when compared to single dose.

E. ANTI-APOPTOTIC AGENT PROTECTS AGAINST VIBRATION-INDUCED CELLULAR DAMAGE IN VITRO

Given the in vivo effect of Z-VAD-FMK on graft outcomes, we tested whether apoptosis inhibitor would protect cell viability and proliferation in response to mechanical vibration in H9C2 myoblast cells. Cell viability did not change

when assessed immediately after mechanical vibration using trypan blue exclusion (data not shown), but was significantly decreased 48 hours later compared to non-vibrated controls (**Suppl. Fig. 2**). When cells were cultured in the presence of Z-VAD-FMK at a range of concentrations (1, 5, 10, or 25uM), viability in the face of vibrational stimulation was protected in a dose-dependent manner up to 10uM. However, there was no difference between vibrated controls and the highest Z-VAD-FMK dose (25uM), likely reflecting cytotoxicity.

IV. DISCUSSION

Our group has worked to innovate the transport, monitoring, and logistics of donor organ transport to improve patient access to transplant and minimize CIT for shipped organs. Our first ever shipment of organ by UAV provided proof-of-principle that human organs can be safely transported by UAV, suggesting that it may be possible to move donor organs directly from donor to recipient facilities once technologies and regulations allow [4]. In order to study the differential effects of various modes of organ transport, we also developed a novel sterile sensor and information technology platform [4], [5]. This new technology revealed that human organs experience different environmental exposures depending on how they are transported. Critically, we observed large discrepancies in vibrational stress between fixed-wing aircraft, ground, and UAV-based transport [4], [5]. Recapitulating the minimum vibrational force a transported tissue might experience in flight (4 Hz) using a mouse heart transplant model, we found that pre-transplant vibration of *ex vivo* allografts was associated with apoptosis, intracellular actin disorganization, and accelerated graft failure.

Donor organs are now being shipped longer distances to intended recipients, with an average kidney travel distance of 706 miles [4]. Farther shipment correlates with longer shipment times and subsequently, prolonged CITs. Donor livers transported greater than 200 miles had a significantly longer CIT than those transported shorter distances (13 hours to 6.1 hours, respectively) [3]. Each additional hour of CIT resulted in reduced liver graft and patient survival during the first year post-transplant (3.4% increase in graft loss/hour) [2]. Furthermore, increased shipping time itself correlates with a greater risk of DGF as well as increased risk of acute rejection and graft loss within the first year [22], [23]. Extended liver transport times of more than 12 hours significantly increased the rate of primary nonfunction compared to shorter times (9.2% vs 1.8%, respectively) [3]. Similarly, when comparing distant versus locally procured liver allografts in Spain, not only was CIT increased, but postoperative bilirubin levels (3.17 mg/mL local vs 4.33 mg/mL distant) and alkaline phosphatase levels (83.95 mg/mL local vs 95.38 mg/mL distant) increased as well [24]. Similarly, a retrospective analysis of pediatric heart transplants between 1987 and 2008 found a negative association between CITs greater than 3.5 hours and allograft survival at 6 months, but not with graft loss beyond this time point [25]. Yet, a retrospective analysis of primary heart transplants performed in the United States between 2000 and 2013 found that increasing donor organ transport distance failed to correlate with perioperative complications, rejection episodes, or 1- and 5-year patient survival. This was despite a linear relationship between shipment distance and CIT (median CIT ranging from 3.1 to 7.5 hours), although severity status correlated with shorter transit distances and decreased donor age correlated with farther distances [26]. These studies reinforce that allograft outcomes are multifactorial and extend beyond CIT or transport time alone, likely including but not limited to allocation algorithms, surgeon preference, and transport environment.

Our data suggests that vibrational forces trigger myocardial apoptosis, contributing to worse allograft outcomes. This aligns with recent work showing that human-induced pluripotent stem-differentiated retinal tissue underwent transport-mediated apoptosis with intensified expression of apoptotic factor cleaved caspase3, p53, NFκB, and TNFα [27]. Future studies will benefit from comparative single cell RNA transcriptomic analysis of vibrated and non-vibrated murine hearts to ascertain whether vibration forces act on cardiac myocytes alone or also on resident immune cells. This type of analysis would help clarify the mechanism underlying the described post-vibration phenotype. Resident dendritic cells and macrophages are known contributors to cardiac tissue homeostasis and repair [28]. In particular, changes in the immunologic transcriptome would help to clarify whether vibration directly triggers cell death or if it induces a pro-inflammatory state, subsequently preventing the recipient immune system from becoming tolerized to the donor allograft. These gene signatures could also be tested against biopsies from human donor hearts following transport with recorded vibration levels.

The observed myocardial cell death and subsequent poor allograft outcomes were ameliorated by the administration of Z-VAD-FMK, an anti-apoptotic pan-caspase inhibitor. Given that the effect of Z-VAD was incomplete, further study of this vibration-induced apoptotic mechanism may refine our ability to treat and protect donor allografts, as well as improve recipient post-transplant recovery. In this study, donor animals were treated with Z-VAD prior to recovery of cardiac allografts, mimicking a clinical scenario of donor-pretreatment for deceased donor heart recovery. This presents the potential application of a therapeutic additive to preservation solutions to reduce the effects of environmental vibration. These data reinforce the importance of measuring environmental stressors on donor organs in transit as well as the benefit of donor pre-treatment as a means for improving recipient outcomes.

Hypothermic and normothermic perfusion devices have already paved the way for monitoring several non-vibration environmental forces. Indeed, hypothermic kidney pumps have allowed for organ temperature and flow dynamics tracking [29]. Liver perfusion devices also provide laboratory data such as blood gas measurements, temperature, and mean arterial pressure [30]. However, there remains an unmet need for more integrated and comprehensive systems that incorporate measurements of physiologic parameters and environmental stressors on donor organs between explant and implant.

This is among the first reports describing the potential negative impact of travel-related environmental stressors on whole donor organs. These initial findings in our murine transplant model suggest that CIT consists of more than just time. Indeed, the time between explant and implant of a transplantable organ includes exposure to environmental stressors such as vibration, pressure, temperature, salinity, and acidity. At present, few if any of these metrics are measured in transported organs. This study suggests that an enhanced understanding of the mechanism underlying shipment environment effects on donor organs could improve our approach to their care during transport. This is a particularly timely study given longer distances organs must travel as allocation systems evolve. While this study does not suggest organs should not travel farther, it does suggest that there remains work be done in terms of improving shipment practices.

V. DISCLOSURE

Dr. Scalea holds patents in organ preservation and monitoring through the University of Maryland. As a result of science published and described herein, Dr. Scalea founded MediGO, Inc. and MissionGO, Inc., companies which increase access to transplants using efficiency-oriented and innovative transportation models.

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