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# The Effect of Increasing Donor Age on Myocardial Ischemic Tolerance in a Rodent Model of Donation After Circulatory Death

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**Background.** Hearts from older donors or procured via donation after circulatory death (DCD) can alleviate transplant waitlist; however, these hearts are particularly vulnerable to injury caused by warm ischemic times (WITs) inherent to DCD. This study investigates how the combination of increasing donor age and pharmacologic supplementation affects the ischemic tolerance and functional recovery of DCD hearts and how age impacts cardiac mitochondrial respiratory capacity and oxidative phosphorylation. **Methods.** Wistar rats (12-, 18-, and 24-mo-old) were subjected to DCD with 20-min fixed WIT. Hearts were procured, instrumented onto a Langendorff perfusion circuit, flushed with Celsior preservation solution with or without supplementation (glyceryl trinitrate [GTN]/erythropoietin [EPO]/zoniporide [Z]) and perfused (Krebs-Henseleit buffer, 37°C Langendorff 30-min, working 30-min). Cardiac functional recovery of aortic flow (AF), coronary flow (CF), cardiac output (CO), and lactate dehydrogenase release were measured. Native heart tissue (3-, 12-, and 24-mo) were assessed for mitochondrial respiratory capacity. **Results.** Unsupplemented 18- and 24-month DCD hearts showed a 6-fold decrease in AF recovery relative to unsupplemented 12-month DCD hearts. GTN/EPO/Z supplementation significantly increased AF and CO recovery of 18-month DCD hearts to levels comparable to supplemented 12-month hearts; however, GTN/EPO/Z did not improve 24-month DCD heart recovery. Compared to 12-month heart tissue, 24-month hearts exhibited significantly impaired mitochondrial oxygen flux at complex I, II, and uncoupled maximal respiration stage. **Conclusions.** Reduced ischemic tolerance after DCD was associated with increasing age. Pharmacologic supplementation improves functional recovery of rat DCD hearts but only up to age 18 months, possibly attributed to a decline in mitochondrial respiratory capacity with increasing age.

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## INTRODUCTION

An aging population from rising life expectancy is now common to virtually all developed and most developing countries.<sup>1</sup> The resulting median age increase poses added burden on healthcare systems and further complexity to human organ transplantation with increasing numbers of older recipients and donors with more comorbidities.<sup>2</sup> The concomitant advances in heart failure management together with significantly improved survival have a direct impact on transplantation needs and greater demands on the continued scarcity of quality donor hearts. These factors have led to the introduction of extended criteria donor categories including marginal brain dead (MBD) and donation after circulatory death (DCD) to meet recipient demand and mitigate against attrition on transplant waiting lists.

Based on current standard criteria for brain dead donation (DBD), the median donor age is 32 years as reported by the International Heart and Lung Transplant Registry<sup>3</sup> with an upper age limit of 55 years accepted by most transplant centers. Other key criteria include cardiovascular disease and risk factor profile; cardiac function (via transthoracic echocardiography) and inotropic requirement; and the exclusion of blood-borne illnesses and high-risk tumors. Recipient-matched

criteria include blood group, crossmatch compatibility, gender, and size matching.<sup>2,4</sup> In contrast, some transplant centers consider MBD donors with an extended upper age limit of 65 years, high inotropic requirement, cardiac function at the lower end of normal range, and prolonged cold ischemic times (4–6 h).

One of the challenges when considering DCD donors is the ischemic tolerance of the native heart to warm ischemia. DCD hearts represent the more extreme spectrum of marginality, primarily due to the inevitable warm ischemic time (WIT) inherent to the DCD donation pathway. At present, a functional WIT of <30 min is tolerated with subsequent normothermic machine perfusion to assess cardiac viability using a combination of physiological and biomarker parameters (ie, aortic pressure, coronary flow [CF], ECG, lactate concentration in the perfusate), and visual inspection.<sup>5</sup> DCD hearts can withstand these long WIT and maintain viable functional recovery due to supplementation of the cardiac preservation flush using glyceryl trinitrate (GTN) and erythropoietin (EPO)—currently in clinical practice for direct procurement DCD protocols within transplantation units in Australia, the UK, and Belgium.<sup>6–8</sup> Although the DCD donor age is limited to 55 years,<sup>6</sup> it is unclear whether pharmacologic supplementation can be of benefit for hearts from older DCD donors.

Preclinical rodent and porcine models of DCD heart transplantation have typically utilized young animals.<sup>9–11</sup> We have previously investigated the effect of pharmacologic conditioning with GTN, EPO, and the sodium–hydrogen exchanger inhibitor zoniporide (Z) on the cardiac recovery of older DBD rodent hearts and observed that supplemented 18-month-old DBD hearts exhibited functional recovery to levels observed in younger DBD rodent hearts.<sup>12</sup> However, the effect of pharmacologic supplementation on the functional recovery of older DCD hearts has not been studied. Here, we investigate the effect of aging on the ischemic tolerance of the heart in a rodent DCD model and test whether pharmacologic supplementation of the preservation flush with a combination of GTN+EPO+Z can enhance functional recovery. Further to this, given that these pharmacologic supplements can influence mitochondrial processes,<sup>13,14</sup> we investigate mitochondrial function in hearts from older rodents.

## MATERIALS AND METHODS

### Animals

Male Wistar rats were sourced from Charles River Laboratories (Kingston, NY). Animals were aged at the Animal Resources Centre (ARC; Canning Vale, Western Australia) for 12-, 18-, and 24-month-old studies, transferred to the BioCORE animal facility (Victor Chang Cardiac Research Institute, Darlinghurst, Australia), and allowed to acclimatize for at least 7 days before experimental studies. Based on studies correlating rat to human age,<sup>15</sup> 12-, 18-, and 24-month-old animals were selected to represent human 30-, 45-, and 60-years of age. An increased incidence of soft tissue tumors and skin disease was observed in the 18- and 24-month groups (n = 5 and 6, respectively), and when associated with irreversible weight loss ≥20%, these animals were culled and excluded from the study. A total of 43 animals were used for DCD studies; however, 8 animals were excluded (Table 1) due to injury of the carotid artery during cannulation or technical issues when cannulating onto the ex vivo perfusion circuit.

**TABLE 1.**

### Experimental groups and exclusions for DCD study

Groups	Numbers used	Exclusions	Final numbers used
12C	6	0	6
12CS	8	2	6
18C	6	0	6
18CS	8	2	6
24C	8	3	5
24CS	7	1	6
Total	43	8	35

C, Celsior only group; CS, Celsior supplemented with GTN/EPO/zoniporide; DCD, donation after circulatory death; EPO, erythropoietin; GTN, glyceryl trinitrate.

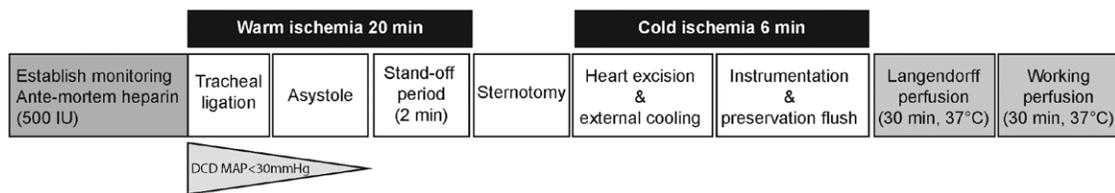
Cohorts of 3-month-old animals for mitochondrial studies were sourced from the ARC. The study was approved by the institutional animal ethics and research committee (Garvan Institute, Animal Research Authority #15/32), and all handling and management of the rodents were compliant with the *Guidelines to Promote the Wellbeing of Animals Used for Scientific Purposes* (National Health and Medical Research Council, Australia) and the *Guide for the Care and Use of Laboratory Animals* (National Institute of Health, Bethesda, MD).

### Surgical Preparation

Animals were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg; Cenvet Australia, Kings Park, Australia) and 0.15 mL of xylazine (20 mg/mL; Provet, Eastern Creek, Australia). Anesthesia was maintained with inhaled 1% isoflurane with supplemental oxygen. Pulse oximetry was used to monitor oxygen saturation and heart rate. Once sufficiently anesthetized as assessed by the absence of withdrawal reflex to a painful stimulus, carotid artery cut-down was performed, the artery exposed and cannulated with a Millar pressure catheter (ADInstruments Inc., Bella Vista, Australia) for continuous blood pressure monitoring. The trachea was exposed and encircled using a 2-0 silk tie.

### Donation After Circulatory Death Protocol

A clinically relevant DCD protocol was established using a rodent model (Figure 1) to closely mimic events during withdrawal of life support (WLS) in the clinical setting.<sup>16</sup> Rodents were subjected to DCD via asphyxiation after surgical preparation under general anesthesia. Once hemodynamics stabilized, a laparotomy was performed, 500 IU of heparin injected into the renal vein, and the trachea was ligated marking the initiation of WLS. A fixed WIT of 20 min was observed for all animals. Upon the completion of the 20-min WIT, a sternotomy was performed, the pulmonary veins were ligated, and the lungs excised followed by the swift excision of the heart. The heart was immersed in ice-cold Celsior preservation solution (Genzyme, Naarden, the Netherlands) and the aorta and pulmonary artery fashioned for instrumentation on an ex-situ Langendorff perfusion circuit. The aorta was cannulated and the heart flushed with 100 mL of ice-cold Celsior preservation solution at a flow rate of 20–30 mL/min administered via the pressure line. Hearts were flushed with either unsupplemented Celsior (C) or supplemented (CS) with 5 U/mL recombinant human EPO- $\alpha$  (Eprex; Janssen-Cilag, North Ryde, Australia), 0.1 mg/mL GTN (Hospira Australia Pty Ltd,



**FIGURE 1.** Rodent DCD protocol. Representative schematic indicating the key steps in the rodent DCD protocol. DCD, donation after circulatory death; MAP, mean arterial pressure.

Mulgrave, Australia), and 1  $\mu\text{mol/L}$  Z (Pfizer Inc, Groton, CT). Left atriotomy was performed as a vent to prevent LV distension and to facilitate cannulation via the left atrial appendage for functional assessment during working mode reperfusion. In addition to the fixed WIT, 2 other timepoints were considered: asystolic-to-cardioplegia delivery time (AP) commencing after the prescribed stand-off period until the initiation of the cold preservation flush; and the cardioplegia-to-reperfusion time (CR) from initiation of preservation flush until the commencement of Langendorff perfusion.

After preservation flush, Langendorff reperfusion was commenced for a period of 30 min followed by 30 min of the working mode using oxygenated Krebs-Henseleit perfusate (composition [mM]: NaCl 118; KCl 4.7;  $\text{MgSO}_4$  1.2;  $\text{KH}_2\text{PO}_4$  1.2;  $\text{NaHCO}_3$  25;  $\text{CaCl}_2$  1.4; glucose 11; pH 7.3–7.4, 37°C) under constant preload and after-load conditions. CF was measured at regular 5-min intervals including baseline during the working mode. Aortic flow (AF), heart rate (HR), and mean arterial pressure (MAP) were measured continuously during working mode. The ex-situ circuit and model of functional assessment have been previously described.<sup>17,18</sup> The study was terminated if animal death occurred before the initiation of DCD withdrawal, there was an injury to the left atrial appendage during cannulation, and failure of cannulation.

### Measurement of Non-Donation After Circulatory Death Heart Baseline Hemodynamics

As a comparison group, hearts were excised from 12-, 18-, and 24-month-old rats that were not subjected to the DCD protocol. Animals were anesthetized as described above, 500 IU heparin administered via the renal vein, the heart excised and immediately immersed in ice-cold preservation solution before instrumentation onto the rig, and Langendorff perfused for 15 min then working mode for 30 min. Baseline hemodynamic measurements of AF, CF, and cardiac output (CO) were recorded during working mode reperfusion.

### Measurement of Lactate Dehydrogenase Release

Lactate dehydrogenase (LDH) release was measured from coronary effluent samples collected during working mode reperfusion using the TOX7 assay kit (Sigma-Aldrich, St. Louis, MS) as per the manufacturer's instruction. LDH efflux was normalized to CF [(abs<sup>450 nm</sup> – abs<sup>690 nm</sup>/CF  $\times$  100)] as previously described.<sup>17</sup>

### Mitochondrial Functional Assessment of Native Heart Tissue

Sections of LV-free wall native tissue from separate 3-, 12-, and 24-month-old rat cohorts (not subjected to DCD or perfusion) were collected in BIOPS buffer (composition [mM]:  $\text{CaK}_2$  EGTA 2.77;  $\text{K}_2$ EGTA 7.23;  $\text{Na}_2$ ATP 5.77;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  9.56;

Taurine 20;  $\text{Na}_2$  phosphocreatine 15; imidazole 20; dithiothreitol 0.5; MES hydrate 50; pH 7.0), and dissected into  $\sim 2$ –4 mm cardiac fiber bundles. Fiber bundles were incubated for 10 min at 4°C in Mitochondrial Respiratory (MiR05) buffer (composition [mM unless specified otherwise]: EGTA 0.5;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  3; taurine 20;  $\text{KH}_2\text{PO}_4$  10; HEPES 20; D-sucrose 110; BSA 1 g/L; lactobionic acid 60) containing 280 U/mL catalase. After incubation,  $\sim 2$ –3 mg/wet weight individual fiber bundles were used for high-resolution respirometry analysis (Oroboros Oxygraph2-k, Oroboros Instruments, Austria). The Substrate-Uncoupler-Inhibitor-Titrations protocol was carried out at 37°C using the following substrates ( $\mu\text{M}$ ): malate 2; octanoyl-carnitine 0.2; adenosine di-phosphate (ADP) 4–20; glutamate 10; succinate 20; carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone (FCCP) 0.5, 1.0, 1.25; rotenone 0.5; and antimycin A 2.5. The respiratory states measured were as follows:  $L_N$ : leak respiration in the absence of adenylates,  $P_{\text{ETF}}$ : maximal electron flow via electron-transferring flavoprotein after the addition of ADP,  $P_{\text{CI}}$ : submaximal state 3 respiratory capacity specific to complex I after the addition of glutamate and pyruvate,  $P_{\text{MAX}}$ : maximal state 3 respiration after second ADP injection, ETS: electron transport system capacity after uncoupling with FCCP,  $P_{\text{CII}}$ : submaximal state 3 respiratory capacity specific to complex III after addition of rotenone, ROX: residual oxygen consumption after addition of antimycin A. Mitochondrial respiratory capacity was corrected for wet weight and baseline values after permeabilization, and the oxygen flux per volume presented as  $\text{pmol/s/mL/mg}$  wet weight.

### Data Analysis

Hemodynamic data were collected and analyzed using Labchart (ADInstruments Inc). Statistical analyses of rat and heart weights, cardiac hemodynamics, and mitochondrial studies were performed using 1-way analysis of variance (ANOVA) with post hoc Tukey's multiple comparisons test, Dunnett's multiple comparisons test, or unpaired t test where specified (GraphPad Prism v8.0.2, GraphPad Software Inc., La Jolla, CA). Statistical analysis of LDH studies was performed using repeated-measures 3-way ANOVA for serial LDH release combined with factorial ANOVA for age and treatment as independent variables using StatView for Windows, version 5.0 (SAS Institute Inc, Cary, NC).

## RESULTS

### Baseline Cohort Characteristics and Cardiac Hemodynamics

Compared to 12-month-old rodents, there was a significant increase in body weight at 18- and 24 months; however, there were no significant differences in heart weight between the different age groups (Figure 2A and B). By comparison, the weight of 3-month hearts was  $2.52 \pm 0.12$  g ( $n = 8$ ) and

significantly lower than all 3 older cohorts ( $P < 0.05$ , data not shown), indicating that heart weight increased in rats beyond 3 months. The observed AF recovery of older hearts not subjected to fixed WIT was 36–46 mL/min (Figure 2C). There were no significant differences in AF, CF, and CO between 12-, 18-, and 24-month-old hearts when hemodynamics were measured in the absence of warm ischemia and immediately after heart procurement (Figure 2C–E).

### Key Time Intervals During Withdrawal of Life Support

All age groups were subjected to a 20-min fixed WIT based on pilot data from our laboratory demonstrating excellent recovery with 12 min fixed WIT but no AF recovery when WIT was extended to 25 min (data not shown). There were no significant differences in the agonal time (time from WLS to asystole) between the age groups: 12-month  $6.9 \pm 1.4$  min (mean  $\pm$  SD), 18-month  $7.3 \pm 1.9$  min, and 24-month  $7.8 \pm 1.3$  min. There were no differences in AP times between the age groups: 12-month  $6.1 \pm 0.9$  min, 18-month  $6.1 \pm 1.4$  min, and 24-month  $5.4 \pm 0.9$  min. There were also no differences in CR times between the age groups: 12-month  $7 \pm 1.3$  min, 18-month  $6.6 \pm 1.2$  min, and 24-month  $6.8 \pm 1.2$  min.

### Functional Recovery of Hearts After Fixed Warm Ischemic Time With and Without Pharmacologic Supplementation

Hearts from the 12-month-old cohort that were subjected to fixed WIT demonstrated an AF recovery of  $\sim 13$  mL/min; however, there was an age-related decline in AF recovery of 18- and 24-month-old hearts to  $\sim 2$  mL/min, with a significant overall difference in AF with age ( $P = 0.0411$ , Figure 3A). There were no significant differences in CF recovery between the age groups (Figure 3B) but there was a trend for reduced CO recovery with increasing age ( $P = 0.0681$ , Figure 3C). These data indicate that there is an age-related decline in cardiac functional recovery of DCD hearts.

We next sought to determine whether pharmacologic supplementation of the cardiac preservation solution administered after the fixed WIT could improve the functional recovery of aged DCD hearts. Analysis of AF recovery showed that supplementation of 12-month hearts had a slight increase in AF recovery; however, in 18-month hearts, supplementation resulted in a significant improvement in AF recovery ( $P = 0.0198$ , Figure 3A). In contrast, the poor AF recovery in 24-month-old hearts was not rescued with supplementation (Figure 3A). There were no significant changes in CF recovery (Figure 3B). Supplementation had an overall significant improvement in CO ( $P = 0.0341$ ) although specifically there was a trend towards significantly improved CO recovery within the 18-month group ( $P = 0.0731$ , Figure 3C). These data indicate that as donor age increases, the decline in cardiac functional recovery of DCD hearts can potentially be restored with pharmacologic supplementation; however, the benefits of supplementation are negated in 24-month hearts.

### Analysis of Lactate Dehydrogenase Efflux as an Indicator of Cellular Injury

To determine whether pharmacologic supplementation would have an impact on myocardial injury after fixed WIT, coronary effluent samples collected during the working mode

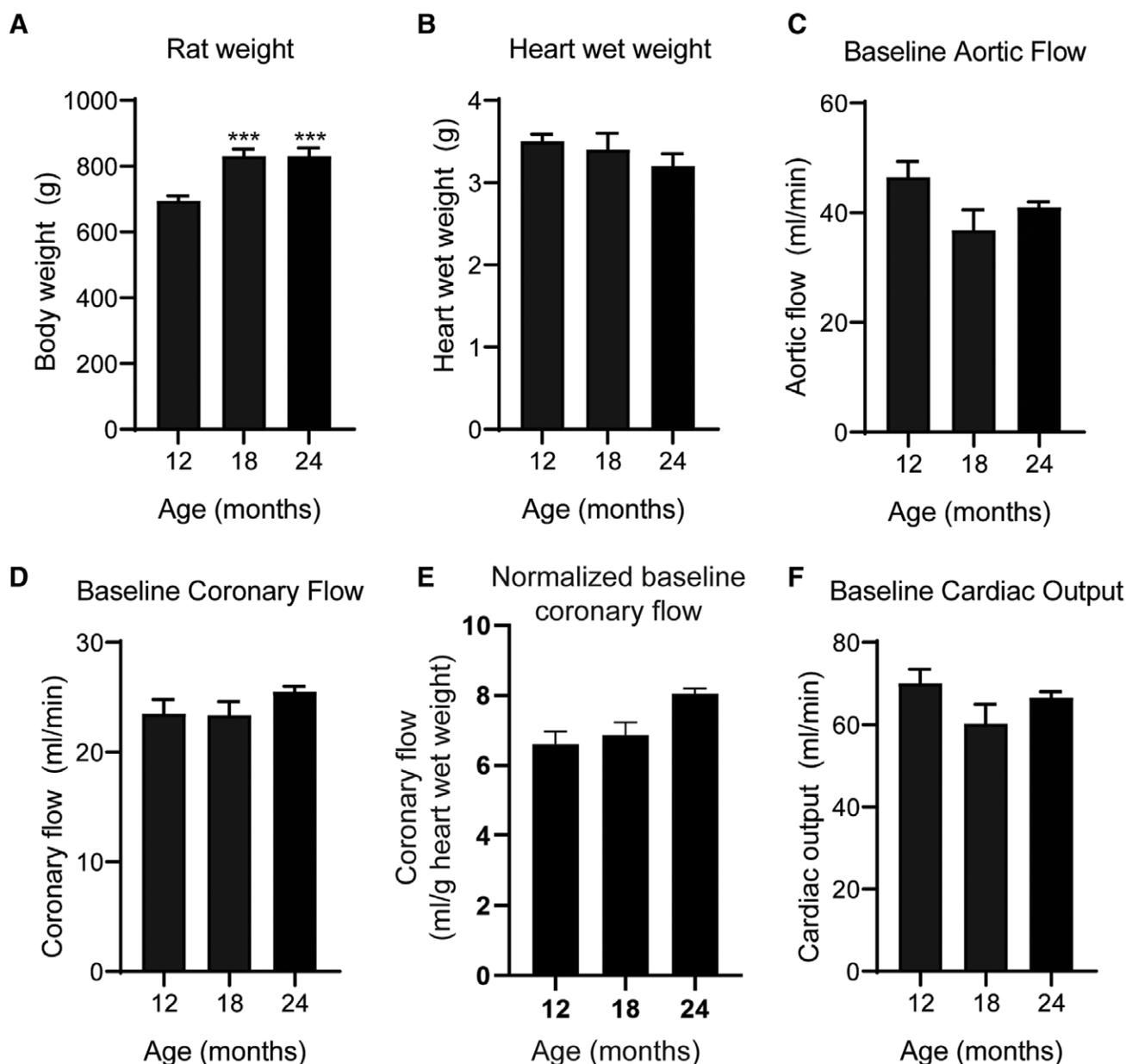
reperfusion were assessed for LDH release. Compared to unsupplemented hearts, LDH efflux after 1 min of working mode reperfusion showed a trend for reduced LDH efflux in 12-month and 18-month supplemented hearts but the trend for increased LDH efflux in 24-month supplemented hearts (Figure 4A); however, these trends did not approach statistical significance. Similar trends for reduced LDH efflux in supplemented 12- and 18-month hearts but increased LDH efflux in 24-month supplemented hearts were observed after 15 min (Figure 4B) and 30 min (Figure 4C) of working mode reperfusion. These data demonstrate that functional improvements achieved by supplementation are limited by the age of the donor's heart since hearts beyond 18 months do not respond to pharmacologic supplementation.

### Mitochondrial Respiratory Capacity in Aged Cardiac Tissue

To elucidate whether the age-related decline in cardiac functional recovery after DCD could be due to impaired mitochondrial function, high-resolution respirometry was used to compare the mitochondrial respiratory capacity of young 3-month native cardiac tissue with native 12- and 24-month-old tissue (Figure 5). Overall, cardiac tissue from the 12-month group demonstrated oxygen flux profiles similar to 3-month tissue at each of the respiratory states measured. However, compared to both 3- and 12-month native heart tissue, tissue from 24-month-old hearts showed a significant decline in oxidative phosphorylation at complex I, reduced maximal state 3 respiration measured after the addition of succinate, reduced ETS capacity measured after uncoupling with FCCP, and reduced respiratory capacity of complex II after inactivation of complex I with rotenone. These data demonstrate that aged cardiac tissue exhibits poor mitochondrial respiratory capacity compared to younger counterparts.

## DISCUSSION

This study shows that DCD hearts exhibit an age-related decline in ischemic tolerance and functional recovery which can be restored with the use of pharmacologic supplementation in rodent donors up to the age of 18 months, the equivalent of a 45-year-old human. The use of pharmacologic supplementation in the cardiac preservation solution improved ischemic tolerance and cardiac functional recovery in a rodent DCD model for the young and “middle-age” cohort (corresponding to human 30- and 45-y-old) but shows limited effect in older cohorts (corresponding to human 60 y old). The present study also demonstrated an age-related decline in mitochondrial respiration, consistent with the observed decline in ischemic tolerance. Our laboratory has previously shown that aged DBD rats have increased susceptibility to ischemia-reperfusion injury induced by brain death and prolonged hypothermic storage.<sup>12</sup> Our current rodent data support the notion that age-related changes in ischemic tolerance influence the degree of functional recovery and adds to previous studies from our laboratory which demonstrate that pharmacologic supplementation can increase the ischemic tolerance in young DCD donors and older BD donors.<sup>9,12</sup> The current data are limited to ischemic tolerance of the donor's heart and may not reflect similar patterns seen in other organ systems. However, current clinical data suggest worse survival in recipients of



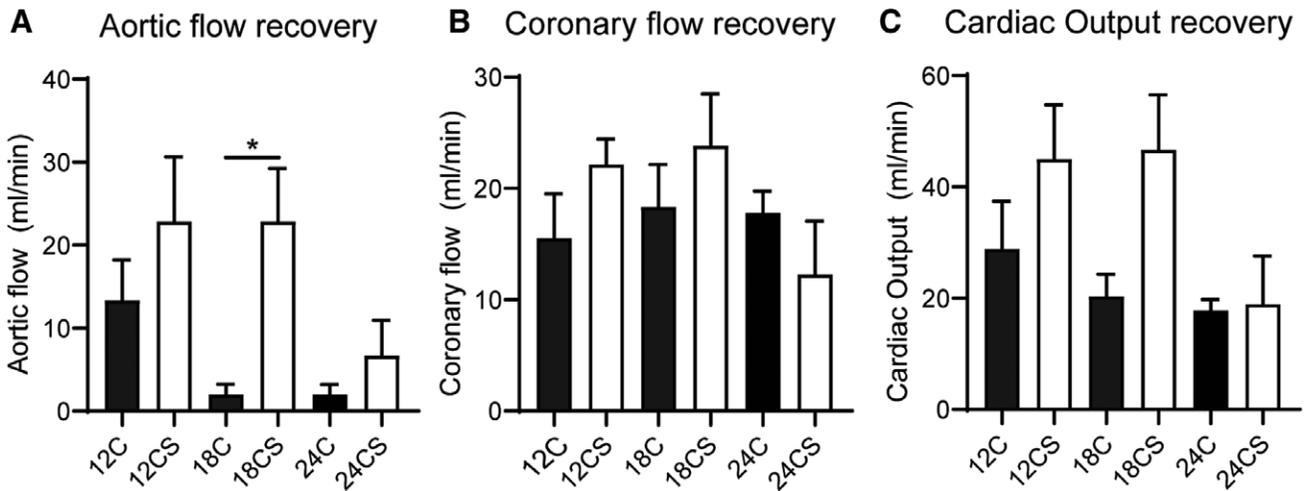
**FIGURE 2.** Baseline characteristics of aged rats and hemodynamics at the end of 30min working mode reperfusion in the absence of warm ischemia. A, Rat body weight,  $n=11-12$ ; (B) heart wet weight, 12-mo and 18-mo  $n=3$ , 24-mo  $n=8$ ; (C) baseline aortic flow, 12-mo and 18-mo  $n=6$ , 24-mo  $n=2$ ; (D) baseline coronary flow, 12-mo and 18-mo  $n=6$ , 24-mo  $n=2$ ; (E) baseline coronary flow normalized to heart wet weight, 12-mo and 18-mo  $n=6$ , 24-mo  $n=2$ ; (F) baseline cardiac output, 12-mo and 18-mo  $n=6$ , 24-mo  $n=2$ . Bars represent mean  $\pm$  SEM; 1-way analysis of variance with Tukey multiple comparisons test. \*\*\* $P \leq 0.0002$  vs 12-mo group.

older donor organs (ie, liver, kidneys, pancreas, heart, and lung) independent of recipient age group.<sup>19</sup>

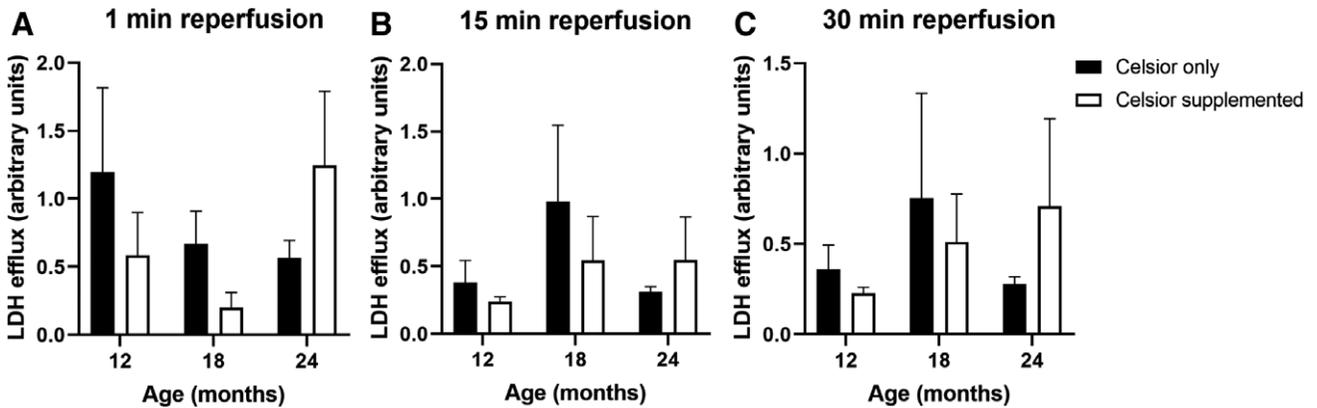
The reduced ischemic tolerance and responsiveness of older hearts to pharmacologic supplementation may be due to impaired mitochondrial respiratory capacity, as demonstrated by the reduced oxygen flux profiles of 24-month-old hearts compared to 3- and 12-month-old hearts. Given that the heart is one of the organs that contain the highest coenzyme Q (CoQ) content,<sup>20</sup> and CoQ plays a crucial role in electron transport between complex I-II and II-III of the electron transport chain, one possibility is that the reduced respiratory capacity observed in 24-month-old hearts is associated with CoQ deficiency.<sup>21</sup> Previous reports have demonstrated an age-related decline in mitochondrial respiration; however,

the extent of this decline varies between reports and shows discrepancies between rodent strains.<sup>22-24</sup> In addition, previous studies vary with respect to whether particular mitochondrial subpopulation, interfibrillar subpopulation or total isolated mitochondria are analyzed.<sup>23-25</sup> The mitochondrial function studies within the present study consist of all mitochondrial populations within the cardiac tissue but do not take into account factors such as mitochondrial density or cell numbers.

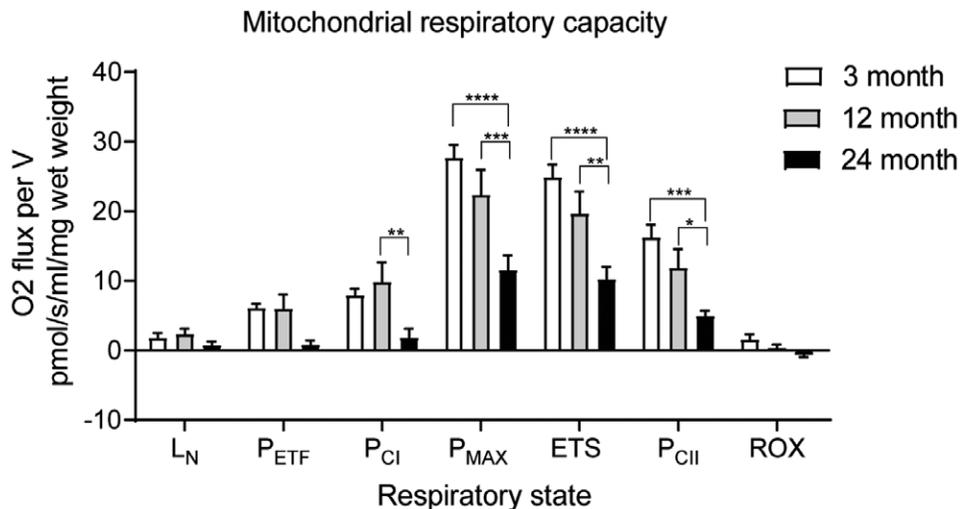
One caveat of this study is that mitochondrial respiratory capacity was examined in native heart tissue but not in heart tissue subjected to DCD with or without pharmacologic conditioning supplements. However, we have previously found the addition of GTN or cariporide (another NHE inhibitor), either



**FIGURE 3.** Cardiac functional recovery of unsupplemented (C, solid bars) and supplemented (CS, open bars) aged donation after circulatory death hearts at the end of working mode reperfusion. (A) Aortic flow recovery; (B) coronary flow recovery; (C) cardiac output recovery. Bars represent mean  $\pm$  SEM; 2-way analysis of variance with Sidak multiple comparisons test comparing unsupplemented vs supplemented groups within each age group. \* $P = 0.0198$ ; all groups  $n = 6$  except 24-mo unsupplemented  $n = 5$ . C, Celsior only group; CS, Celsior supplemented with GTN/EPO/zoniporide; EPO, erythropoietin; GTN, glyceryl trinitrate.



**FIGURE 4.** LDH efflux of unsupplemented (solid bars) or supplemented (open bars) hearts during working mode reperfusion after (A), 1 min; (B), 15 min; and (C), 30 min. Bars represent mean  $\pm$  SEM;  $n = 4$ –6 per group. LDH, lactate dehydrogenase.



**FIGURE 5.** Mitochondrial respiratory capacity from 3-mo (open bars), 12-mo (gray bars) and 24-mo (black bars) old rat hearts. Bars represent mean  $\pm$  SEM. Two-way analysis of variance with Tukey multiple comparisons test; 3-mo  $n = 4$ , 12-mo  $n = 6$ , 24-mo  $n = 5$ . \*\*\*\* $P < 0.0001$ ; \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; and \* $P < 0.03$ . ETS, electron transport system capacity;  $L_N$ , leak respiration in the absence of adenylates;  $P_{CI}$ , submaximal state 3 respiratory capacity specific to complex I;  $P_{CII}$ , submaximal state 3 respiratory capacity specific to complex III;  $P_{ETF}$ , maximal electron flow via electron-transferring flavoprotein;  $P_{MAX}$ , maximal state 3 respiration; ROX, residual oxygen consumption; V, volume.

singly or together during arrest and storage of donor rat hearts resulted in cardioprotective signaling favoring mitophagy activation (ERK and Bcl2 phosphorylation) and maintenance of mitochondrial transition pore closure post-reperfusion (STAT3 and ERK phosphorylation) which were crucial for functional recovery of the donor heart.<sup>26</sup> In addition, previously unrecognized GTN reductase activity of mitochondrial aldehyde dehydrogenase has been demonstrated to be responsible for bioactivation of GTN, by reduction to NO and further conversion to S-nitrosothiols,<sup>27</sup> potentially contributing to mitochondrial protection. Nitrosylation of a key Cys residue on complex I of the electron transport chain can transiently prevent oxidant formation via reverse electron transport through complex I as a result of succinate accumulation during warm ischemia,<sup>28–30</sup> such as after WLS. The loss of ischemic tolerance may be related to mitochondrial dysfunction and we have shown a decline in age-related mitochondrial capacity. The limited effects of pharmacologic supplementation in hearts from older donors may be due to an increased susceptibility of mitochondrial permeability transition pore (mPTP) opening since aged cardiomyocytes exhibit reduced time to mPTP opening in response to an apoptotic stimulus<sup>22</sup> and are more susceptible to mPTP opening after calcium overload.<sup>31</sup> Current clinical supplementations aim to inhibit mPTP opening; however, perhaps the efficacy of supplementation is reduced in older hearts. It is also possible that the increased susceptibility to ischemia in older donors, and lack of responsiveness to pharmacologic supplementation, may be due to downregulation of current known protective pathways.<sup>32,33</sup>

There are some limitations of this study to consider. The current study investigates the effects of ischemia on donor's hearts in an ex-situ setting but does not take into account the posttransplant milieu of the recipient. This study has also solely used hearts from aged male rodents and the effects of supplementation on aged female rodent hearts have not been examined. However, our clinical translation of our DCD protocol suggests that there would be minimal sex differences. Indeed, as yet we have not observed severe primary graft dysfunction in any recipient of a heart from a female DCD donor.<sup>6</sup> Another major limitation is the feasibility of aging rodent studies due to the high financial costs of animal maintenance to account for attrition rates. In the current study, we were unable to assess mitochondrial function in the 18-month cohort due to a lack of additional animals at this particular time point. The clear differences in mitochondrial function between 12 months and 24 months animals indicate an age-related decline in mitochondrial function; however, whether a reduction in mitochondrial respiratory capacity is evident in 18-month animals compared to 12 months is still unknown.

The implications from our current data are 2-fold. First, as aging is common to both the donor and recipient populations, the effect of dwindling ischemic tolerance in older donors may impact on the recovery of the donor's heart. Indeed, a large UNOS registry examining the interaction between donor age and ischemic time in human heart transplant recipients showed that ischemic tolerance declined as the age group increased across 3 age terciles: <20 years, 20–33 years, and >34 years.<sup>34</sup> Although in the present study, senescent markers were not shown, the age groups from the UNOS registry<sup>34</sup> would not be considered as senescent, yet the impact of increasing age on tolerance to ischemia-reperfusion injury is clear. Indeed, the average age of the oldest tercile in the

study by Russo et al<sup>34</sup> was 44 years old, which corresponds closely to the 18-month-old rats in the present study which also displayed a reduced ischemic tolerance. Second, the data from this study raise the question of whether the current age limit for clinical DCD heart utilization (55 y in our clinical DCD heart transplant program<sup>6</sup>) is appropriate given that the benefits of currently available pharmacologic supplements are negated in older donors within the present study.

Although upregulation of protective mechanisms using pharmacologic strategies is effective, the current rodent model suggests that in the elderly, there is irreversible attenuation of these pathways rendering these hearts at risk to irreparable ischemic injury. In conclusion, this study shows an age-related decline to ischemia-reperfusion injury in rodent DCD hearts and suggests continued caution in the clinical use of older DCD hearts for transplantation; however, further understanding of the human DCD heart is required before extending the donor age limit.

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