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Efficacy of mechanical postconditioning following warm, global ischaemia depends on circulating fatty acid levels in an isolated, working rat heart model[†]

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Abstract

OBJECTIVES: The number of heart transplantations is limited by donor organ availability. Donation after circulatory determination of death (DCDD) could significantly improve graft availability; however, organs undergo warm ischaemia followed by reperfusion, leading to tissue damage. Laboratory studies suggest that mechanical postconditioning [(MPC); brief, intermittent periods of ischaemia at the onset of reperfusion] can limit reperfusion injury; however, clinical translation has been disappointing. We hypothesized that MPC-induced cardioprotection depends on fatty acid levels at reperfusion.

METHODS: Experiments were performed with an isolated rat heart model of DCDD. Hearts of male Wistar rats ($n = 42$) underwent working-mode perfusion for 20 min (baseline), 27 min of global ischaemia and 60 min reperfusion with or without MPC (two cycles of 30 s reperfusion/30 s ischaemia) in the presence or absence of high fat [(HF); 1.2 mM palmitate]. Haemodynamic parameters, necrosis factors and oxygen consumption (O_2C) were assessed. Recovery rate was calculated as the value at 60 min reperfusion expressed as a percentage of the mean baseline value. The Kruskal–Wallis test was used to provide an overview of differences between experimental groups, and pairwise comparisons were performed to compare specific time points of interest for parameters with significant overall results.

RESULTS: Percent recovery of left ventricular (LV) work [developed pressure (DP)–heart rate product] at 60 min reperfusion was higher in hearts reperfused without fat versus with fat (58 ± 8 vs 23 ± 26%, $P < 0.01$) in the absence of MPC. In the absence of fat, MPC did not affect post-ischaemic haemodynamic recovery. Among the hearts reperfused with HF, two significantly different subgroups emerged according to recovery of LV work: low recovery (LoR) and high recovery (HiR) subgroups. At 60 min reperfusion, recovery was increased with MPC versus no MPC for LV work (79 ± 6 vs 55 ± 7, respectively; $P < 0.05$) in HiR subgroups and for DP (40 ± 27 vs 4 ± 2%), dP/dt_{max} (37 ± 24 vs 5 ± 3%) and dP/dt_{min} (33 ± 21 vs 5 ± 4%; $P < 0.01$ for all) in LoR subgroups.

CONCLUSIONS: Effects of MPC depend on energy substrate availability; MPC increased recovery of LV work in the presence, but not in the absence, of HF. Controlled reperfusion may be useful for therapeutic strategies aimed at improving post-ischaemic recovery of cardiac DCDD grafts, and ultimately in increasing donor heart availability.

Keywords: Cardiac ischaemia–reperfusion • Mechanical postconditioning • Circulating fatty acids • Donation after circulatory determination of death • Heart transplantation

INTRODUCTION

Approaches to reduce cardiac ischaemia–reperfusion injury have been the subject of many years of intense research activity, yet effective and robust clinical reperfusion strategies are lacking.

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Mechanical postconditioning (MPC), defined as brief, intermittent periods of re-occlusion applied during the first minutes of reperfusion, has been demonstrated to be cardioprotective in preclinical studies; however, clinical benefits remain controversial and incompletely characterized [1–10]. In both *in vivo* and *ex vivo* animal models of regional cardiac ischaemia, MPC is recognized to reduce infarct size and arrhythmia development; however, the effects after global ischaemia need to be clarified. In isolated, working rat heart preparations, MPC was indeed reported not to improve cardiac output (CO) after global, normothermic ischaemia [11], whereas it increased recovery of LV developed pressure (DP) and CO after global, hypothermic (4°C) ischaemia [12], and improved recovery of dP/dt_{max}, but not dP/dt_{min} or DP following low-flow ischaemia with pacing [13].

Importantly, high levels of circulating fatty acids at the time of reperfusion are known to be detrimental to post-ischaemic haemodynamic recovery [14, 15]. This is particularly relevant for the heart as circulating fatty acid level is elevated during cardiac surgery [16]. A high circulating level of fat leads to increases in fatty acid uptake and oxidation, which, in turn, is believed to impart its negative effects on post-ischaemic recovery through the inhibition of glycolysis and the even greater inhibition of glucose oxidation [17, 18]. Importantly, the exaggerated imbalance between rates of glycolysis and glucose oxidation increases lactate production and tissue acidosis, thereby exacerbating calcium overload at reperfusion.

Although postconditioning and circulating fat levels are recognized individually to impact on post-ischaemic cardiac recovery, interplay between the two remains to be characterized. Interestingly, MPC has been demonstrated to promote glucose metabolism through elevated glucose uptake (via increased GLUT 4 translocation), glycolysis and glycogenolysis, whereas interventions that inhibit glycolysis or glycogenolysis attenuate cardioprotective effects of MPC, suggesting that metabolic effects play a key role in MPC-induced cardioprotection [19, 20].

We have developed and worked over the last years with an isolated, global ischaemia, working rat heart model, in order to simulate conditions of donation after circulatory determination of death (DCDD) [21–23]. Our model has been designed to study procurement and reperfusion strategies that could possibly enable optimization of post-ischaemic recovery and graft evaluation. In addition, our studies also aim at identifying early markers that could predict post-transplantation heart function [21, 22]. One critical aspect in the development of this model was to provide conditions that lead to an intermediate level of post-ischaemic haemodynamic recovery; if the model would allow a high recovery (HiR) rate, the benefits of reperfusion strategies could not be quantified, and conversely if too low, heart damage may be too severe to permit improvements. In our model, with normothermic ischaemia, all hearts recover almost completely after 20 min ischaemia and they homogeneously show no recovery after 30 min ischaemia [23]. Under similar conditions with intermediate ischaemic times, recovery rates range between 20 and 80% depending on the parameter measured, and tend to form two subgroups: HiR and low recovery (LoR; data not shown) [21]. As such, we have chosen 27 min as the ischaemic time for the current work.

In the present study, we aimed to investigate whether MPC effectively improves post-ischaemic haemodynamics in our isolated working rat heart model of DCDD. We hypothesized that MPC-induced effects on recovery depend on the presence of fatty acids in the reperfusion solution, and that haemodynamic parameters measured during early reperfusion provide information that aid in the prediction of subsequent recovery.

METHODS

Materials

Albumins from bovine serum and palmitic acid were obtained from Sigma-Aldrich (Buchs, Switzerland). All other chemicals were acquired from Merck (Darmstadt, Germany).

Ethics statement

All animal experimental procedures were performed in accordance with the guidelines of the European Convention for Animal care as well as local laws and policies, and were approved by the Swiss animal welfare authorities and state veterinary office (Authorization numbers: BE101/12 and BE11/11). All surgeries were performed under anaesthesia, and all efforts were made to minimize animal suffering.

Isolated heart preparation and perfusion protocol

Isolated hearts of adult, male Wistar rats were perfused in the working mode, as previously described [21, 24]. Briefly, adult male Wistar rats, housed under standard conditions with unlimited access to food (standard laboratory diet *ad libitum*) and water, were anaesthetised using 100 mg/kg of ketamine (Narketan®, Vetoquinol AG, Bern, Switzerland) and 10 mg/kg of xylazine (Xylapan®, Vetoquinol AG) via an intraperitoneal injection. Hearts were excised and immediately placed in ice-cold, modified Krebs–Henseleit bicarbonate (KHB) buffer containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$, 1.25 mM $CaCl_2{\cdot}7H_2O$, 25 mM $NaHCO_3$ and 11 mM glucose. Hearts were perfused with this buffer via the cannulated aorta at a constant pressure of 60 mmHg. The left atrium was then cannulated, and a micro-tip pressure catheter (Scisense, London, Canada) was inserted into the left ventricle (LV) via the mitral valve.

Ex vivo heart perfusion was designed to simulate DCDD conditions (Fig. 1). First, hearts underwent a baseline perfusion in a working mode for 20 min. During this period, heart function was evaluated to exclude hearts with a LV DP–heart rate (HR) product (<23 000 mmHg beats min^{-1}). Then, hearts were subjected to 27 min of global, no-flow, normothermic (37°C) ischaemia; for this, perfusion lines were clamped and the hearts were immersed in a tissue bath containing energy substrate-free KHB maintained at 37°C and bubbled with 95% N_2–5% CO_2. Finally, hearts were reperfused for 60 min with KHB supplemented with 1.2 mM palmitate (high fat; HF) or not (no fat; NF). Reperfusion was initiated via the aortic cannula in the unloaded (Langendorff) mode at a constant pressure of 60 mmHg for 10 min. Additionally, hearts were randomized to receive MPC, consisting of two cycles of 30 s reperfusion and 30 s ischaemia (MPC+) or not (MPC–). Following the 10-min unloaded reperfusion, reperfusion was switched to working mode for the remaining 50 min. KHB buffer was maintained at 37°C, and oxygenated with 95% O_2–5% CO_2 throughout the aerobic baseline perfusion and reperfusion periods. During working-mode perfusion, preload (PL) and

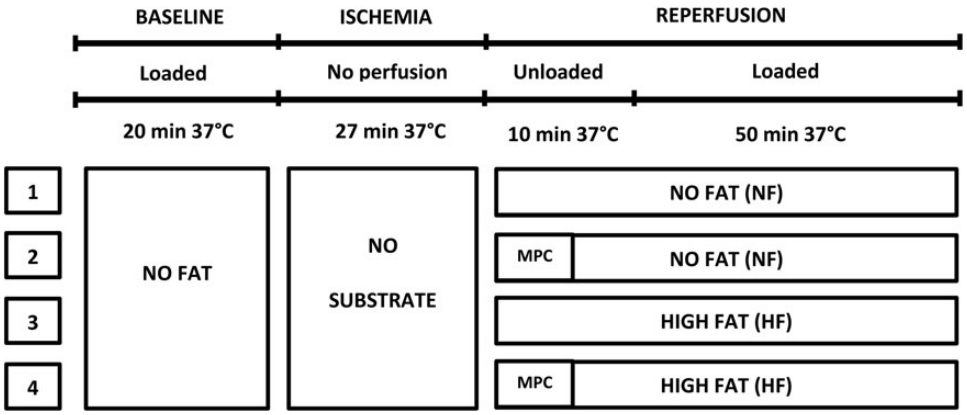

Figure 1: Perfusion protocol. Hearts ($n = 42$) were perfused for 20 min in an aerobic working mode, subjected to global, no-flow ischaemia for 27 min at 37°C, and reperfused for 60 min with or without MPC (two cycles of 30 s reperfusion/30 s ischaemia). Hearts were reperfused with buffer containing either no fat or high fat (1.2 mM palmitate).

afterload (AL) pressures were maintained at 11.5 and 80 mmHg, respectively.

Data collection

LV pressure was measured with a pressure catheter (Scisense); perfusate flows in PL and AL lines were measured using flowsensors and flowmeters (Transonic System, Inc., Ithaca, NY, USA). These parameters were continuously recorded using a PowerLab data acquisition system (ADInstruments, Spechbach, Germany). The following haemodynamic parameters were assessed: HR (beats min^{-1}), LV DP (mmHg), maximum and minimum first derivatives of LV pressure (dP/dt_{max} and dP/dt_{min} [mmHg s^{-1}]), LV work (DP * HR; mmHg beats min^{-1}), coronary flow (CF, [PL–AL; ml min^{-1}]) and CO (PL; ml min^{-1}). Recovery rate was calculated as 60 min reperfusion time points expressed as a percentage of the mean baseline value (average of 10 and 20 min values).

Samples of coronary effluent and circulating buffer were taken at various time points: during the pre-ischaemic period at 0, 10 and 20 min; and during reperfusion at 0, 3, 5, 10, 20, 40 and 60 min. These samples were used for quantification of necrosis markers and metabolic parameters.

Lactate dehydrogenase production was assessed using the Roche MODULAR P800 analyser (Roche Diagnostics Corp., Indianapolis, IN, USA). Troponin-T production was assessed with the Roche MODULAR E170 analyser (Roche, Basel, Switzerland) and an electro-chemiluminescence-immunoassay analyser (Roche). Oxygen consumption (O_2C) was determined using the Cobas b 123 blood-gas analyser (Roche).

Data analysis

Unless stated otherwise, values are reported as mean ± SD. Stata (version 12.0, StataCorp, College Station, TX, USA) was used for data analysis. The median test was used to provide an overview of differences between experimental groups. Pairwise comparisons were performed to compare specific time points of interest for parameters with significant overall results. Spearman correlations were used to investigate associations between percent recovery of LV work at 60 min reperfusion and predictive parameters at 10 min reperfusion. Multiple comparisons and tests were corrected with the sequentially rejective Bonferroni procedure [25]. With this procedure, families of parameters, rather than individual parameters, are considered in the correction of *P*-values to prevent an overly severe correction [25]. Three families of parameters were considered: baseline physiological measures, haemodynamic measures and biochemical measures. All *P*-values were two-sided, adjusted for multiple comparisons and reported after correction. Corrected *P*-values of <0.05 were considered statistically significant.

RESULTS

A total of 42 hearts were included in this study. The post-ischaemic recovery of HF-reperfused hearts was generally lower than NF-reperfused hearts (Fig. 2). Compared with NF hearts, recovery of LV work in HF hearts was more variable, but not in a continuous manner; hearts tended to recover relatively well (>40%) or very poorly (<40%; Fig. 3A). Accordingly, a cut-off value of 40% recovery LV work was used to separate hearts into HiR and LoR subgroups for further analysis (Fig. 3B).

Baseline characteristics

Rat heart and body weights, as well as haemodynamic parameters during the pre-ischaemic period, are presented in Table 1. No statistically significant difference between groups was observed with the exception of LV work, which was significantly lower in the HF/MPC−/HiR group versus NF/MPC− group ($P < 0.01$).

Haemodynamic function

As mentioned above, recovery was generally lower in HF hearts compared with NF hearts; recovery of LV work at 60 min reperfusion was 23 ± 26% for MPC− and 34 ± 30% for MPC+ groups (Fig. 2). Recovery of LV work at 60 min reperfusion was significantly lower in HF versus NF hearts only in the absence of MPC (23 ± 26 vs 58 ± 8%, $P < 0.01$).

Haemodynamic recovery during reperfusion is presented as absolute values and percent recovery at 60 min (Fig. 4 and Table 2). All haemodynamic parameters remained unchanged with the

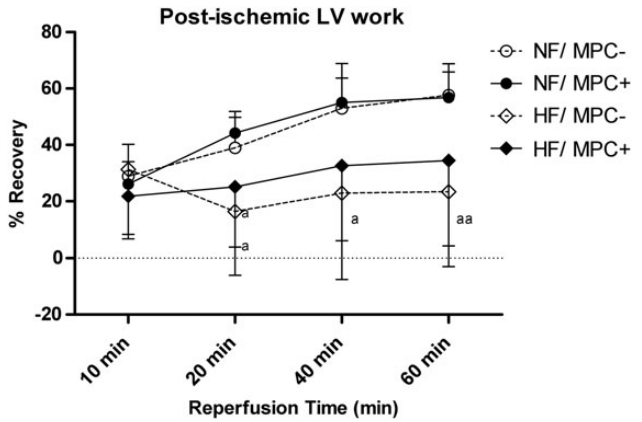

Figure 2: Post-ischaemic LV work. Percent recovery LV work for no-fat (NF) and high-fat (HF) reperfusion groups with and without MPC. $^{a}P < 0.05$, $^{aa}P < 0.01$, versus corresponding NF group. LV: left ventricular; MPC: mechanical postconditioning.

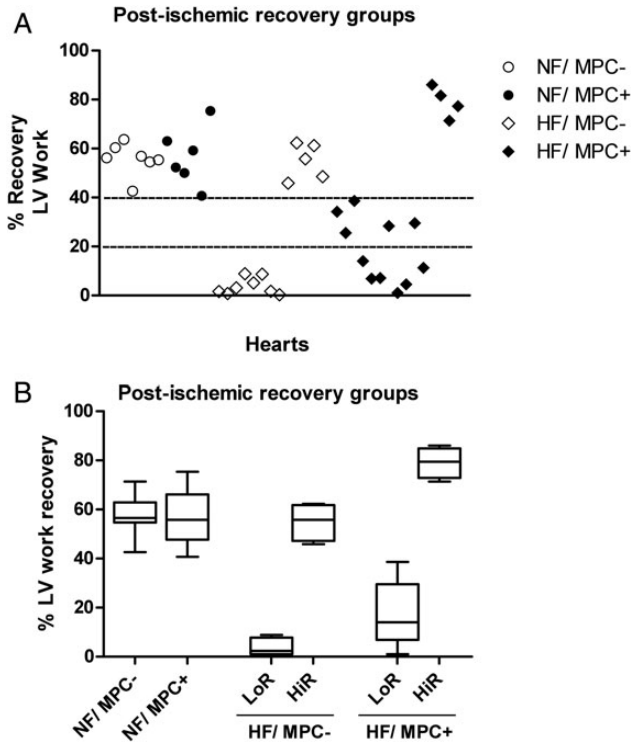

Figure 3: Distribution of post-ischaemic LV work recovery. Percent recovery LV work after 60 min reperfusion presented as scatter (**A**) and box (**B**) plots. Variability is relatively low in the no-fat reperfusion group. In contrast, for high-fat reperfusion groups, variability is high and clear divisions between recovery subgroups become evident. A division between low (LoR) and high (HiR) recovery subgroups, respectively, for both MPC– and MPC+ hearts occurs at approximately 40% recovery, and is represented by a broken line (**A**).

application of MPC in NF hearts. Recovery of LV work at 60 min reperfusion was 58 ± 8% for MPC– and 57 ± 12% for MPC+ groups. In contrast, haemodynamic recovery improved with MPC in HF hearts. In LoR subgroups, 60 min reperfusion recoveries were significantly greater in MPC+ versus MPC– groups for DP (40 ± 27 vs 4 ± 2%), dP/dt_{max} (37 ± 24 vs 5 ± 3%) and dP/dt_{min} (33 ± 21 vs 5 ± 4%; $P < 0.01$ for all). In addition, percent recovery of DP was significantly lower than the corresponding NF value in the absence, but not in the presence, of MPC (Table 2). Similarly, in HiR subgroups, percent recovery of LV work at 60 min reperfusion was significantly greater in MPC+ versus MPC– groups (79 ± 6 vs 55 ± 7, respectively; $P < 0.05$).

Release of cell death markers and O_2C are presented in Fig. 5A and B, respectively. With our isolated heart preparation, it is the heart itself that generates the perfusion pressure when in working, loaded mode. Therefore, during reperfusion, when heart function varies among groups, parameters that depend on coronary flow must be interpreted with care. As such, we have chosen to present markers of necrosis and O_2C, which are both dependent on coronary flow, at the 10-min reperfusion time point, when the perfusion pressure is fixed at 60 mmHg (unloaded mode). Troponin-T production was significantly increased in all HF groups compared with NF ($P < 0.01$ for all). A similar pattern was observed for lactate dehydrogenase, although not statistically significant. O_2C was significantly lower in LoR subgroups versus corresponding NF groups (918 ± 485 vs 1855 ± 306 mmHg ml^{-1} min g wet^{-1} for MPC+ and 1112 ± 583 vs 2000 ± 213 mmHg ml^{-1} min g wet^{-1} for MPC– $P < 0.01$ for both). Interestingly, among HiR groups, recovery of O_2C was significantly greater for MPC+ versus MPC– hearts (2755 ± 211 vs 1621 ± 676 mmHg ml^{-1} min g wet^{-1} for MPC–, $P < 0.01$).

Predictive parameters

Haemodynamic parameters measured during early, unloaded reperfusion correlated significantly with percent recovery of LV work after 60 min reperfusion in HF hearts (Supplementary Table 1 and Fig. 6). Notably, LV work and DP at 10 min reperfusion demonstrated strong correlations with percent recovery of LV work after 60 min reperfusion for correlations with both all HF hearts and HF/MPC+.

DISCUSSION

We report that postconditioning can improve haemodynamic recovery following a prolonged period of global, normothermic ischaemia, and that its effects are dependent on reperfusion energy substrate levels. In hearts reperfused without HF, overall post-ischaemic haemodynamic recovery was significantly higher than HF reperfused hearts, but MPC provided no additional benefit. Conversely, and although post-ischaemic haemodynamic function was reduced with HF reperfusion, MPC provided a significant benefit for these hearts, suggesting that MPC can overcome some detrimental effects of HF. Furthermore, among HF-reperfused hearts, early reperfusion haemodynamic parameters correlated with recovery 60 min after ischaemia. These findings provide insights into key mechanisms underlying reperfusion strategies, and may contribute towards initial steps in the development of clinical protocols for reperfusion strategies aimed at optimizing post-ischaemic recovery of cardiac DCDD grafts.

In this study, we investigated the effects of HF during reperfusion on MPC-induced changes in haemodynamic recovery. Interestingly, MPC did not alter haemodynamic recovery when hearts were reperfused in the absence of fat. These findings are consistent with those of Van Vuuren *et al.* [11], who investigated the effects of MPC in the isolated, working rat heart after a period of global ischaemia.

With HF reperfusion, haemodynamic recovery was lower than with NF reperfusion and two subgroups emerged during reperfusion according to haemodynamic function. In general, compared

Table 1: Baseline characteristics

	No-fat		High-fat			
	MPC−	MPC+	MPC−/LoR	MPC−/HiR	MPC+/LoR	MPC+/HiR
Number of hearts (*n*)	8	6	8	5	11	4
BW (g)	337 ± 50	349 ± 45	392 ± 29	358 ± 84	382 ± 55	333 ± 80
HW (g)	1.94 ± 0.34	1.95 ± 0.23	2.32 ± 0.31	2.01 ± 0.48	2.22 ± 0.42	1.77 ± 0.29
LV work (mmHg beats min^{-1})	33 492 ± 3741	33 295 ± 4975	29 377 ± 3894	25 802 ± 2805[aa]	30 729 ± 4101	27 739 ± 1320
HR (beats min^{-1})	271 ± 26	269 ± 23	264 ± 30	235 ± 23	261 ± 28	249 ± 21
DP (mmHg)	123 ± 9	124 ± 20	113 ± 11	110 ± 8	118 ± 6	112 ± 11
CF (ml min^{-1})	20.1 ± 4.7	19.3 ± 3.8	21.7 ± 5.2	22.2 ± 8.6	22.4 ± 3.9	18.4 ± 5.5
CO (ml min^{-1})	48.9 ± 8.0	44.0 ± 13.3	43.1 ± 6.8	35.0 ± 12.8	42.6 ± 10.2	44.6 ± 13.6
dP/dt_{min} (mmHg s^{-1})	−3299 ± 382	−2927 ± 533	−2829 ± 1089	−1925 ± 828	−3050 ± 1152	2658 ± 275
dP/dt_{max} (mmHg s^{-1})	4099 ± 476	4164 ± 1122	3330 ± 1011	2420 ± 1301	3339 ± 1110	3427 ± 313

All parameters are reported as mean ± SD pre-ischaemic values.
BW: body weight; HW: heart weight; HR: heart rate; DP: developed pressure; LV work: left ventricular work (DP–HR product); dP/dt_{min}: minimum first derivative of LV pressure; dP/dt_{max}: maximum first derivative of LV pressure; CF: coronary flow; CO: cardiac output.
[aa]$P < 0.01$: versus corresponding NF group.

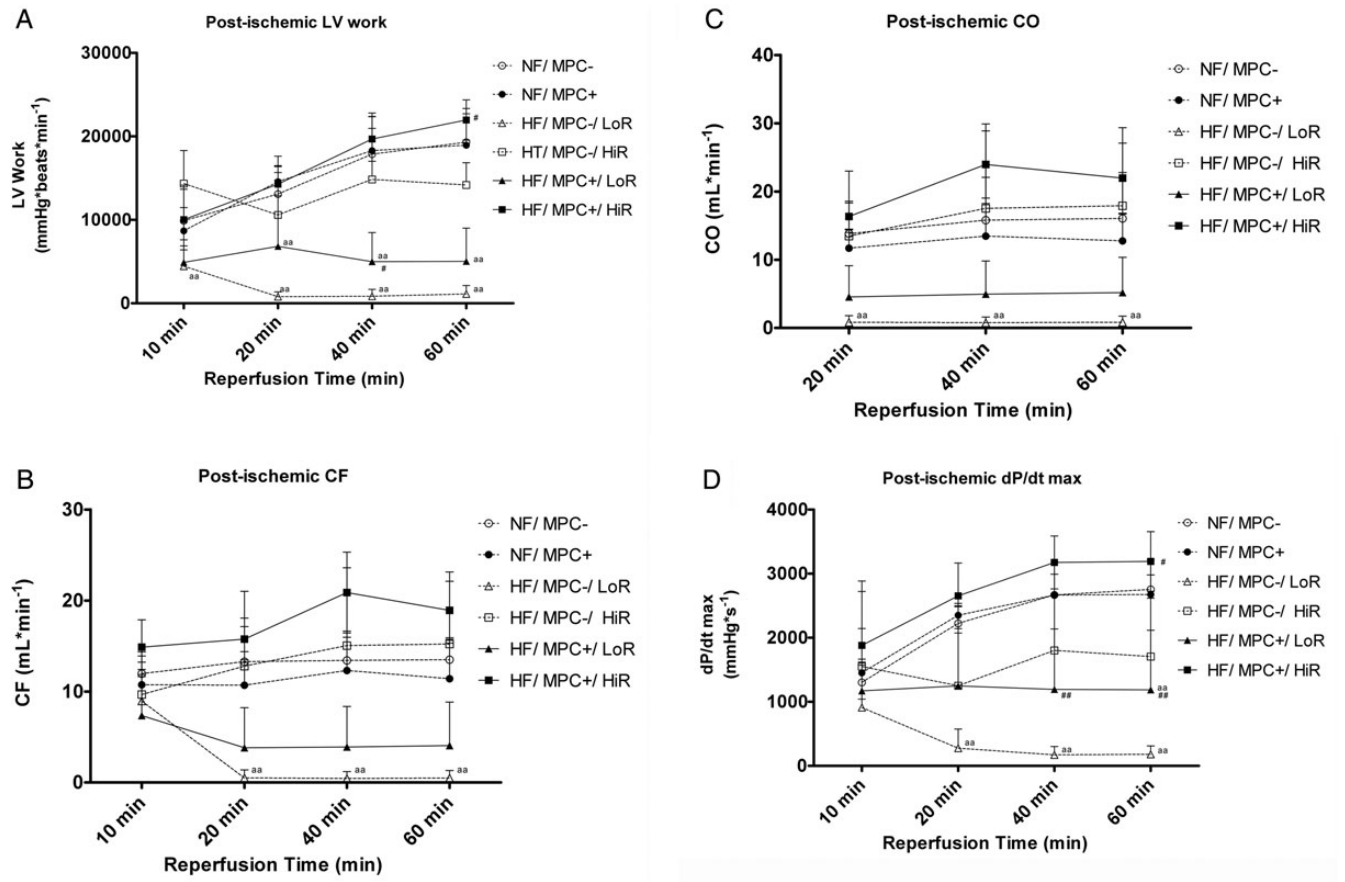

Figure 4: Post-ischaemic LV work. Recovery of haemodynamic parameters during reperfusion for LV work (**A**), coronary flow (CF; **B**), cardiac output (CO; **C**), dP/dt_{max} (**D**) and dP/dt_{min} (**E**). HF: high-fat reperfusion; HiR: high recovery subgroup; LoR: low recovery subgroup; MPC: mechanical postconditioning; NF: no-fat reperfusion. [a]$P < 0.05$, [aa]$P < 0.01$, versus corresponding NF group; [#]$P < 0.05$, [##]$P < 0.01$, versus corresponding MPC− group.

with NF reperfusion, HF conditions resulted in lower post-ischaemic haemodynamic function as previously reported [14, 15, 17, 18]. With HF reperfusion, however, haemodynamic recovery was variable and two subgroups, such as LoR and HiR, became apparent. Importantly, subgroups were not observed among hearts perfused without fat; thus, these subgroups likely represent true biological variation, not technical artefacts arising from the isolated heart preparation or the perfusion technique. To our knowledge, few groups use intraventricular pressure catheters in the isolated working rat heart system; most use pressure transducers

Table 2: Percentage recovery of haemodynamic parameters after 60 min reperfusion

Percent recovery	NF		HF			
	MPC− (*n* = 8)	MPC+ (*n* = 6)	MPC−/LoR (*n* = 8)	MPC−/HiR (*n* = 5)	MPC+/LoR (*n* = 11)	MPC+/HiR (*n* = 4)
LV work (%)	58 ± 8	57 ± 12	4 ± 3^{aa}	55 ± 7	18 ± 13^{aa}	79 ± 6$^{\#}$
Heart rate (%)	82 ± 4	83 ± 14	58 ± 36	94 ± 14	45 ± 20^{aa}	89 ± 4
Developed pressure (%)	71 ± 10	68 ± 7	4 ± 2^{aa}	66 ± 17	40 ± 27$^{\#\#}$	90 ± 11
Coronary flow (%)	64 ± 31	58 ± 10	3 ± 6^{aa}	66 ± 28	21 ± 25^{aa}	106 ± 15
Cardiac output (%)	5 ± 1	4 ± 1	2 ± 2	46 ± 20^{a}	14 ± 14	51 ± 16
dP/dt_{min} (%)	62 ± 13	65 ± 11	4 ± 4^{aa}	62 ± 10	32 ± 21$^{aa, \#\#}$	88 ± 15
dP/dt_{max} (%)	68 ± 14	67 ± 13	5 ± 4^{aa}	70 ± 13	37 ± 24$^{\#\#}$	94 ± 18

All values are reported as mean ± SD.
dP/dt_{max}: maximum first derivative of LV pressure; dP/dt_{min}: minimum first derivative of LV pressure; NF: no fat; HF: high fat; HiR: high recovery subgroup; LoR: low recovery subgroup; LV work: left ventricular work (DP–HR product); MPC: mechanical postconditioning.
$^{a}P < 0.05$, $^{aa}P < 0.01$: versus corresponding NF group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$: versus corresponding MPC− group.

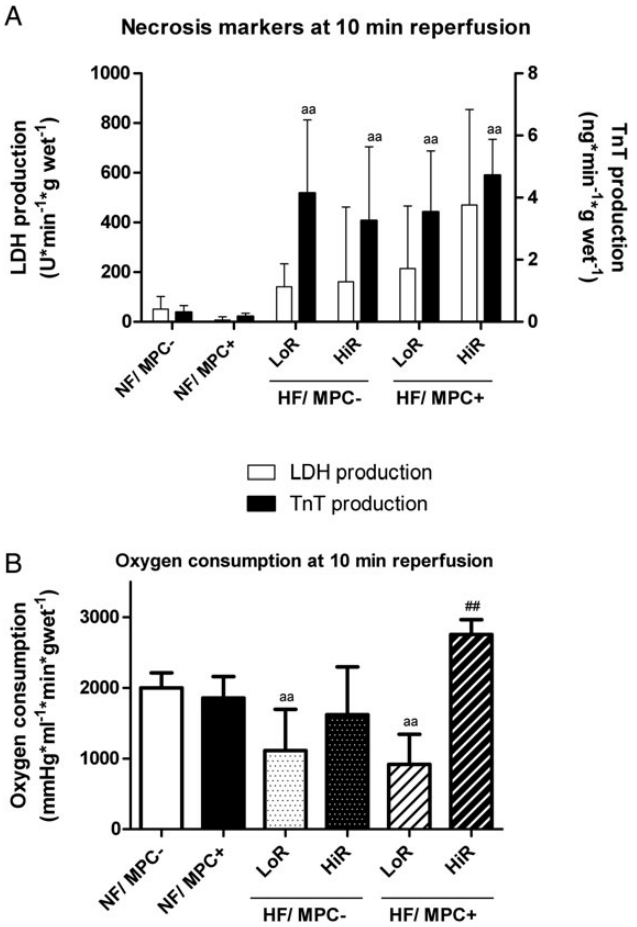

Figure 5: Necrosis markers and oxygen consumption at 10 min reperfusion. Absolute values at 10 min reperfusion are reported for lactate dehydrogenase (LDH) and troponin-T (TnT) production (**A**), as well as oxygen consumption (**B**). $^{aa}P < 0.01$ versus corresponding NF group; $^{\#\#}P < 0.01$ versus corresponding MPC− group.

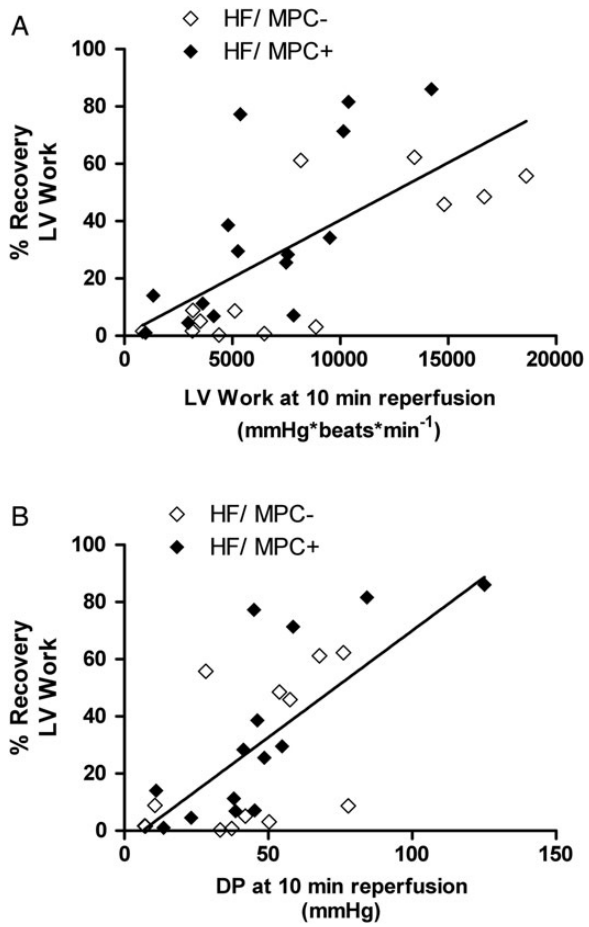

Figure 6: Early reperfusion predictive parameter correlations with 60 min haemodynamic recovery. Scatter plots are presented for predictive parameters that demonstrated statistically significant Spearman correlations with percent recovery of LV work after 60 min reperfusion; LV work (**A**) and developed pressure (DP), (**B**) versus percent recovery of LV work.

in the aortic line. With intraventricular pressure measurements, we obtain precise functional measurements, which may enable us to detect subgroups that are not obvious with pressure transducer measurements. Indeed, we have previously observed a similar pattern in isolated rat hearts in which haemodynamic recovery was measured following global, no-flow ischaemia of varied duration [21]. This corresponds closely to the situation observed in the present study; post-ischaemic recovery was higher in NF groups and variability is low, while the addition of HF during reperfusion led to an overall lower recovery that was associated

with greater variability and subgroups. These findings of subgroups are of particular interest as a model for preclinical investigation into underlying mechanisms of ischaemia–reperfusion injury, given that confounding factors are minimized.

Our data are consistent with the concept that MPC-induced improvements in haemodynamic function depend on increased glucose metabolism. MPC promotes glucose metabolism by stimulating glucose uptake (via increased GLUT 4 translocation), glycolysis and glycogenolysis, and the inhibition of these processes attenuates MPC-induced cardioprotection [19, 20]. These findings suggest that MPC-induced modifications in glucose metabolism contribute to its cardioprotective effects. Increased rates of glycolysis may supply the ATP to maintain K^+/Na^+ activity, thereby limiting Na^+-induced injury arising from Na^+/H^+ exchange glycogenolysis [19, 20]. It is also interesting to speculate that increased rates of glycolysis could promote activation of mitoK (ATP) and mitoK (Ca) channels, providing cardioprotection by reducing excess generation of reactive oxygen species and the Ca^{2+} overload [19]. Given that maximal glucose metabolism would be expected with NF reperfusion in our study, further stimulation by MPC may therefore not be possible, and is reflected by unchanged haemodynamic recovery. In contrast, we speculate that MPC may overcome some of the inhibitory effects of fatty acids on glucose metabolism, and thereby effectively improves haemodynamic recovery.

Measurements of O_2C, but not markers of necrosis, demonstrated a pattern of recovery similar to that for haemodynamic recovery. Interestingly, very similar profiles were observed for O_2C at 10 min reperfusion and recovery of LV work after 60 min reperfusion, indicating that improved post-ischaemic recovery is associated with higher early reperfusion O_2C. In contrast, markers of necrosis did not follow the profile of haemodynamic recovery; at 10 min reperfusion, release of necrosis markers was greater in hearts perfused with, versus without, fat. This finding suggests that necrosis may be a contributing factor to the generally lower recovery of hearts reperfused in the presence of HF, but does not appear to be altered by MPC.

For HF-reperfused hearts, haemodynamic parameters (LV work and DP) measured during early reperfusion correlate with recovery of LV work after 60 min reperfusion. Identification of such parameters, which can be easily and rapidly measured at the time of heart procurement, is particularly relevant for DCDD. Establishment of clinical protocols for the evaluation of transplant suitability and the prediction of post-transplantation contractile function will be a necessary and critical step for progress towards routine heart transplantation with DCDD.

Limitations

Our findings provide clear evidence that energy substrate availability at reperfusion influences MPC-induced cardioprotection. Nonetheless, several limitations to this work persist. For example, hearts subjected to HF reperfusion were divided into subgroups based on post-ischaemic haemodynamic recovery for analysis. The *post hoc* grouping is not ideal and the cut-off value used for subgroup division is somewhat arbitrary. However, given the high variability in post-ischaemic recovery under these experimental conditions (HF reperfusion), subgroups were required to interpret the effects of energy substrate availability on MPC, likely reflecting the complex, multifactor nature of cardiac ischaemia–reperfusion injury. One further limitation of this study is that only two energy substrate conditions were investigated during reperfusion: NF and HF. Given our interesting finding that, in a subgroup of hearts, the combination of high fatty acid levels and MPC led to post-ischaemic haemodynamic recovery that was at least as good as NF hearts, it is tempting to speculate that intermediate levels of fatty acids would provide an even greater increase in recovery when combined with MPC. However, these experiments were not included in the current study, but are currently being investigated in our laboratory. In addition, these results were obtained with an isolated rat heart model; therefore, further preclinical testing is required and approaches must be validated in a larger heart model prior to clinical translation. Furthermore, in a DCDD setting, hearts will be exposed to a second period of ischaemia for transportation, albeit under cold, cardioplegic conditions; therefore, maintenance of benefits obtained with reperfusion strategies applied at the time of graft procurement must be confirmed following storage and transplantation. Finally, our analysis of post-ischaemic recovery was limited to 60 min, and effects of procurement reperfusion strategies on long-term recovery will require additional investigation.

CONCLUSIONS

We report that the cardioprotective efficacy of MPC is greatly influenced by energy substrate availability. Indeed, the application of MPC improved post-ischaemic recovery in hearts reperfused with HF; however it did not alter post-ischaemic recovery in hearts reperfused without fat. Furthermore, we have identified haemodynamic parameters, which may aid in predicting the subsequent recovery of these hearts when measured at the time of procurement. We consider these results to be an early step towards the understanding and identification of a controlled reperfusion protocol aimed at improving post-ischaemic recovery of cardiac DCDD grafts, which may ultimately aid in increasing donor heart availability.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *EJCTS* online.

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APPENDIX. CONFERENCE DISCUSSION

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Dr M. Cikirikcioglu *(Geneva, Switzerland):* Your study looks like a futuristic science fiction idea, heart transplantation from a non-heart-beating donor, but, on the other hand, non-heart-beating organ donation was a dream years before for any organs and today it is a clinical reality for certain transplantations, including kidney, liver, and lung transplantation. We should not forget also that in the past we had many science fiction ideas, including the paper of Carpentier on the French repair, describing 3D echo, degradable annuloplasty rings, but those are the clinical realities today. For that reason, I liked your idea. Even though it is not for routine clinical use, it may help us to expand the organ donation, organ pool in the future for heart transplantation. On the other hand, we use hypothermic myocardial protection today for heart transplantation and in your study you used normothermic myocardial ischaemia. I feel that point is a limitation in your study. What was the reason you chose normothermic ischaemia instead of hypothermic myocardial ischaemia in your study?

Dr Bartkevics: We use normothermic ischaemia in our model because we aim to stimulate the clinical situation of warm ischaemia with DCDD hearts during the hands-off period, prior to organ procurement. This warm period is very important for all DCDD organs, since in the clinics, it will always occur before procurement and reperfusion.

Dr Cikirikcioglu: But still, do you think the organs will be kept as warm ischaemic, or will you give some cold cardioplegic solutions before transplantation for clinical use?

Dr Bartkevics: Actually, with DCDD after circulatory arrest, a hands-off period must be respected, and its length varies from country to country – it may be 10 or 20 minutes, or it may be only 5 minutes. So, during this ischaemic time the heart is warm. We must also take into account the time required for cannulation and reperfusion of the organ after this warm ischaemic hands-off period. In a clinic scenario, the warm ischaemic time would be approximately 20 to 30 minutes, in our model, it was 27 minutes.

Dr Cikirikcioglu: My second question is: if it will be applicable tomorrow, a cardiac transplantation from a non-heart-beating donor in your clinic on a patient based on your study, how will you translate the techniques which you used in your experimental study? Will you transfuse some lipid or high-energy solutions? Will you intermittently declamp the aorta before total declamping? What will be the clinical translations of your results?

Dr Bartkevics: It should remain speculative about what could be used in a future clinical DCDD protocol. However, we now know that circulating levels of fatty acids affect cardioprotective reperfusion strategies, and this could be helpful in identifying an optimal approach for heart preservation. In this step, at reperfusion, we could consider mechanical postconditioning, but, as you can see from our results, heart recovery remains variable and is highly dependent on energy substrate availability. So, for the moment, there is not one clear protocol for clinical translation, but we can say that the circulating levels of fatty acids should be taken into consideration.

Dr Cikirikcioglu: I think you will get more details in the future by using those models.

Dr E. Potapov *(Berlin, Germany):* It is a very interesting idea. How do you see your work in conjunction with the TransMedics system? We can now transport beating hearts, but it still requires some period of ischaemia to put the heart into the system and to start this, approximately 27 minutes. So how is it possible to implement your measurements in this TransMedics system online to predict what will happen with the hearts in 2 or 3 hours?

Dr Bartkevics: We think that these organ transport systems could be very, very useful for the next development steps with our model, and for sure in clinical studies and future clinical practice. For example, we think that, after the period of warm ischaemia, use of these transport systems could enable evaluation of haemodynamic parameters to indicate whether the heart would be suitable for transplantation or not. So, it is both very likely and very promising for heart transplantation with DCDD that these perfusion and transport systems will be incorporated into future clinical protocols.