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ORIGINAL ARTICLE

Mild hypothermia during global cardiac ischemia opens a window of opportunity to develop heart donation after cardiac death

Mathieu Stadelmann,[1,2] Monika Dornbierer,[1] David Clément,[1,3] Brigitta Gahl,[1] Florian Dick,[1] Thierry P. Carrel,[1] Hendrik T. Tevaearai[1] and Sarah Longnus[1]

1 Department of Cardiovascular Surgery, Inselspital, Berne University Hospital and University of Berne, Switzerland
2 Department of Cardiology, University & Hospital, Fribourg, Switzerland
3 Department of Biological Engineering, IUT, University of Auvergne, Clermont-Ferrand, France

Correspondence
Prof. Hendrik Tevaearai, Department of Cardiovascular Surgery, Inselspital, Berne University Hospital, CH-3010, Berne, Switzerland.
Tel.: 41 (0)31 632 12 80;
fax: 41 (0)31 632 97 66;
e-mail: hendrik.tevaearai@insel.ch

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Institution at which research performed: Department of Cardiovascular Surgery, Inselspital, Berne University Hospital and University of Berne, Switzerland

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Summary

Although heart donation after cardiac death (DCD) could greatly improve graft availability, concerns regarding warm ischemic damage typically preclude transplantation. Improving tolerance to warm ischemia may thus open a window of opportunity for DCD hearts. We investigated the hypothesis that, compared with normothermia, mild hypothermia (32° C) initiated after ischemic onset improves cardiac functional recovery upon reperfusion. Isolated, working hearts from adult, male Wistar rats underwent global, no-flow ischemia, and reperfusion (n = 28). After ischemic onset, temperature was maintained at either 37° C for 20 or 30 min or reduced to 32° C for 40, 50, or 60 min. Recovery was measured after 60-min reperfusion. Following normothermic ischemia, recovery of rate-pressure product (RPP; per cent of preischemic value) was almost complete after 20-min ischemia (97 ± 9%), whereas no recovery was detectable after 30-min ischemia. After mildly hypothermic ischemia (32° C), RPP also recovered well after 40 min (86 ± 4%). Markers of metabolism and necrosis were similar in 37° C/20 min and 32° C/40 min groups. Simple reduction in cardiac temperature by a few degrees after the onset of global ischemia dramatically prolongs the interval during which the heart remains resistant to functional deterioration. Preservation of hemodynamic function is associated with improved metabolic recovery and reduced necrosis. The application of mild hypothermia may be a simple first step towards development of clinical protocols for DCD heart recovery.

Introduction

Donation after cardiac death (DCD) represents a currently untapped source of hearts that could radically change the future for an increasing number of patients awaiting cardiac transplantation. In recent years, the gap between supply and demand of donor organs has led to a renewed interest in DCD as a potential source of cardiac grafts. In fact, DCD is already considered as an alternative to donation after brain-death for various organs including kidney, liver, lung, and pancreatic islets. Importantly, it was recently estimated that adoption of DCD in heart transplantation could increase organ supply by 17% for adults [1] and 42% for children [2]. Furthermore, the feasibility of DCD heart transplantation has been clinically demonstrated in recently reported pediatric cases [3]. However, clinical approaches to organ procurement that permit optimized post-transplant cardiac graft function remain to be identified.

In the setting of heart transplantation with DCD, cardiac grafts undergo a period of warm ischemia between cardiac arrest and preservation. DCD in heart transplantation has

not yet been widely adopted, likely as a result of the widespread belief that a period of warm ischemia inevitably triggers irreversible myocardial injury [4] and subsequent postoperative contractile dysfunction. These conditions contrast with traditional multi-organ procurement procedures, in which cardiopulmonary function is maintained in a physiologic range until hearts are perfused with a cold preservation solution, thereby entirely avoiding warm ischemia. However, DCD organs undergo a period of warm ischemia that cannot be avoided, as a 'hands-off' period of 2–10 min is legally imposed before any clinical intervention is authorized [5, 6]. Thus, effective approaches to optimally protect cardiac grafts remain to be established for the particular context, and limitations, surrounding DCD.

Despite its susceptibility to damage, the myocardium is able to withstand limited periods of ischemia; cellular and molecular changes are initially reversible, with prolongation of ischemia inducing permanent injury and cell death [7]. Paradoxically, reperfusion following ischemia also contributes to the cell and organ injury [7, 8]. Therefore, when aiming to develop a strategy for protection of DCD organs, it is useful to distinguish ischemia-induced injury from reperfusion-induced injury; the former depending both on the legally imposed 'hands-off' time and on the technical time required to access the heart for the eventual application of a protective solution. Importantly, several studies have clearly demonstrated that a normal heart retains sufficient integrity for transplantation if warm ischemia is limited to 20–30 min [9–12]. Therefore, a window of opportunity, albeit limited, does exist for useable DCD cardiac grafts and should most probably be exploited.

Similar to the preconditioning principle, approaches applied at reperfusion have been proposed to 'postcondition' the heart and may emerge as powerful strategies for limiting myocardial ischemia–reperfusion injury. Given that reperfusion itself contributes significantly to ischemia–reperfusion injury, and that interventions to protect hearts during DCD can currently be initiated only after the onset of ischemia, actions that limit reperfusion injury, such as postconditioning, constitute very promising approaches. Importantly, an optimal cardioprotective strategy may require a combination of complementary protective strategies – for example, mild hypothermia during ischemia and postconditioning – to best limit the multiple processes induced by cardiac ischemia and reperfusion that lead to irreversible injury.

Mild hypothermia (32° C–35° C) during ischemia is one potentially clinically applicable intervention that could be useful for prolonging ischemic tolerance and providing time to better preserve DCD grafts. Hypothermia, in general, is well recognized as cardioprotective and mild hypothermia has been demonstrated to provide effective anti-infarct protection in several animal studies; albeit, clinical implementation has proven challenging (for reviews see [13, 14]). However, it currently remains unclear to what extent reducing cardiac temperature by a few degrees after the onset of global ischemia may have on cardiac tolerance to injury arising from global, no-flow ischemia. Importantly, if this type of mild hypothermia reduces cardiac damage and/or extends warm ischemic tolerance, it would likely open the door to use of DCD hearts by increasing cardiac graft availability via augmentation of the allowable time between cardio-circulatory arrest and cardioprotective maneuvers. Therefore, and as a first step toward the development of a potential clinical protocol, we investigated the hypothesis that compared with normothermia, mild hypothermia (32° C) initiated immediately after the onset of global, no-flow ischemia, improves cardiac functional, and metabolic recovery upon reperfusion.

Methods

Materials

Bovine albumin (fraction V) was purchased from Sigma-Aldrich (Buchs, Switzerland) and insulin, from Nordisk Pharma (Actrapid HM 100IE/UI/ml, Küsnacht, Switzerland). All other chemicals were obtained from Merck (Darmstadt, Germany).

Ethics statement

All experimental procedures were performed in compliance with the European Convention for Animal Care and approved by the Swiss animal welfare authorities (Authorization Nos: 58/08 and 11/11). All surgery was performed under anesthesia and all efforts were made to minimize suffering.

Isolated heart preparation and perfusion protocol

Hearts (2.0 ± 0.3 g) from anesthetized adult, male Wistar rats were excised and prepared as previously described [15]. From the establishment of the working preparation, hearts were perfused only with the Krebs–Henseleit (KH) buffer containing (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4{\cdot}7H_2O$ 1.2, $CaCl_2{\cdot}2H_2O$ 1.25, $NaHCO_3$ 25, and glucose 11 supplemented with 1.2 mM palmitate, 3% albumin, and 500 μU/ml insulin; this includes the entire period of reperfusion, both unloaded and loaded. Ischemia was initiated by clamping preload- and afterload-perfusate lines and hearts were immersed in a tissue bath containing energy-substrate-free KH buffer bubbled with 95% N_2–5% CO_2, and pre-set to the desired temperature. Five ischemic groups were analyzed: ischemia at 37° C for 20 or 30 min, or ischemia at 32° C for 40, 50, or 60 min (Fig. 1). These differing ischemic lengths were chosen to cover the range

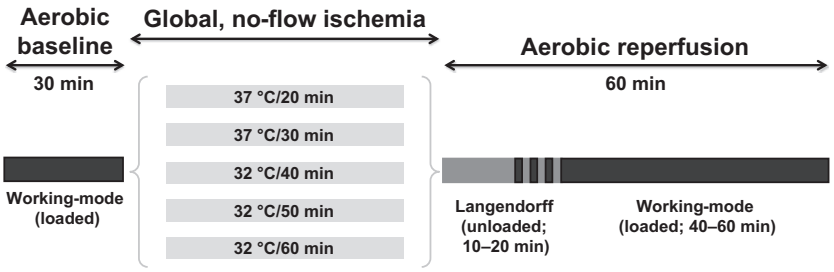

Figure 1 Perfusion protocol Isolated rat hearts were subjected to 30-min, working-mode (loaded), aerobic perfusion, followed by global, no-flow ischemia and 60-min reperfusion. Ischemia was either normothermic (37° C) for 20 or 30 min or mildly hypothermic (32° C) for 40, 50, or 60 min. During reperfusion, hearts were perfused in a retrograde manner (unloaded) for approximately one-third ischemic time and subsequently loaded for the remaining time.

across the time point at which postischemic functional recovery significantly declined according to preliminary results (data not shown). Reperfusion was performed initially in a partially unloaded mode (aortic pressure of 60 mmHg; no preload) for approximately one-third of ischemic time (10–20 min). We have termed this partially unloaded period as 'unloaded reperfusion'. Following unloaded reperfusion, hearts were switched to working mode for the remainder of the 60-min reperfusion period.

During the working mode, heart rate and peak systolic pressure were recorded by a pressure transducer (Edwards Lifesciences, Irvine, CA, USA) in the afterload line coupled to a high-performance data acquisition system (PowerLab; ADInstruments, Spechbach, Germany). Coronary flow (CF) and afterload flow were measured by timed collection, and samples of coronary effluent and circulating buffer were taken at the following time points: baseline; during the preischemic period at 10, 20, and 30 min; and during reperfusion at 1, 2, 5, 10, 20, 40, and 60 min.

Additional series of hearts were perfused to determine the time between ischemic onset and cardiac arrest or cooling to target temperature of 32° C. To do so, a micro-tip pressure catheter (Millar Instruments, Houston, TX, USA) was inserted, via the left-atrial cannula, through the mitral valve and into the left ventricle and a temperature probe (ADInstruments, Spechbach, Germany) was inserted into the left ventricle via the aortic cannula. Beating time was calculated as the time between clamping of perfusate lines and last heart beat with developed pressure ≥ 2 mmHg. Cooling time was calculated as the time from clamping of perfusate lines until 32° C was reached.

Metabolic parameters

Oxygen partial pressure and lactate were measured in buffer samples with an iSTAT analyzer (Abott, Baar, Switzerland) using CG4+ cartridges (Axonlab, Baden, Switzerland).

Necrosis markers

Troponin T was measured using an ECLIA analyzer (ElektroChemiLumineszenzImmunoAssay; Roche, Basel, Switzerland). Lactate dehydrogenase (LDH) was assessed with a Roche MODULAR P800 analyzer (Roche Diagnostics Corp., Indianapolis, IN, USA).

Data analysis

The rate-pressure product (RPP), corresponding to heart work, was calculated as the product of heart rate and peak systolic pressure. Cardiac output was calculated as the sum of coronary and afterload flows. Percent recovery at the end of 60-min reperfusion was calculated from the mean end-reperfusion value (40 and 60 min) as a percent of mean preischemic value (10, 20, and 30 min). Percent recovery during unloaded reperfusion was calculated as the percentage at 10-min reperfusion of the mean preischemic value.

Oxygen consumption was calculated as follows: (PaO_2–PvO_2)*CF/heart weight, where PaO_2 (mmHg) = arterial (circulating buffer) PO_2, PvO_2 (mmHg) = venous (coronary effluent) PO_2, CF was reported as ml/min, and heart weight was reported as g wet weight.

Statistical analysis

All values are expressed as mean $\pm$ SD. Data were analyzed with SPSS for Windows (version 17.0; SPSS Inc, Chicago, IL, USA). Statistical analysis was performed using an ANOVA with repeated measures, for an overview of effects of time, group, and their interaction. When significant overall results were observed, more specific analyses were performed to compare time points of particular interest with comparisons between groups using *t*-tests. *P*-values were all two-sided, adjusted for multiple comparisons, and reported after correction. Corrected *P*-values <0.05 were considered statistically significant.

Results

Baseline characteristics

During the preischemic period of perfusion, parameters were similar among groups, with the exception of two parameters. Peak systolic pressure was higher in 32° C/50 min and 32° C/60 min ischemic groups vs. 37° C/30 min and 32° C/40 min ischemic groups and cardiac output was lower in the 32° C/40 min ischemic group vs. 32° C/50 min and 32° C/60 min ischemic groups, see Table 1 and Fig. 2 ($P < 0.05$ for all comparisons).

Characterization of model: ischemic arrest and mild hypothermia

After the initiation of ischemia (clamping perfusate lines), target temperature of 32° C within the ventricle was reached after 162 ± 24 s, and hearts continued to beat for 188 ± 26 s at 37° C vs. 142 ± 38 s at 32° C ($n = 6$ per group, P = NS).

Effects of mild hypothermia during ischemia on contractile recovery

Among hearts subjected to ischemia at 37° C, contractile function, as measured by heart RPP, was almost completely restored at 60-min reperfusion following 20 min, but not 30 min of global ischemia. Peak systolic pressure (PSP) and heart rate (HR) were not detectable in any heart after ischemia at 37° C for 30 min; PSP ≤12 mmHg. However, among hearts subjected to ischemia at 32° C, contractile function was largely restored after 40 and 50 min of ischemia, but not 60 min (Fig. 2a). Furthermore, PSP and HR were detectable in four of six hearts subjected to ischemia of 60 min at 32° C. Interestingly, no differences in RPP during reperfusion were measured between the following ischemic groups: 37° C/20 min, 32° C/40 min, or 32° C/50 min. Accordingly, percent recovery was >75% for the 37° C/20 min ischemic group, and >80% for both 32° C/40 min and 32° C/50 min ischemic groups (Fig. 2b). Indeed, percent recovery was <20% for all the hearts in 37° C/30 min and 32° C/60 min ischemic groups.

Although the percentage of recovery was somewhat lower for cardiac output, a similar pattern was observed among ischemic groups (Fig. 2c and d); after 60-min reperfusion, cardiac output recovered to >45% of preischemic values for groups exposed to ischemia at 37° C/20 min, 32° C/40 min, or 32° C/50 min, whereas <10% recovery was observed for groups exposed to ischemia at 37° C/30 min, 32° C/60 min. Indeed, percent recovery of cardiac output was significantly lower in hearts exposed to ischemia at 37° C/30 min or 32° C/60 min compared with all other groups ($P < 0.05$ for all comparisons; Fig. 2d).

Effects of mild hypothermia during ischemia on coronary perfusion

During the initial, unloaded period of reperfusion, for which afterload pressure was maintained at 60 mmHg, coronary flow at 10-min reperfusion was significantly lower in 37° C/30 min group versus all other ischemic groups ($P < 0.01$ for all comparisons; Fig. 3).

Once hearts were re-loaded (i.e., when pressure to perfuse the coronary vasculature entirely dependent on ventricular contraction), coronary flow rapidly dropped in 37° C/30 min and 32° C/60 min ischemic groups, as afterload pressure did not recover to sufficiently perfuse the hearts. At 40- to 60-min reperfusion in 37° C/30 min and 32° C/60 min ischemic groups (hearts that did not recover), coronary flow represented only 17 ± 13% and 22 ± 9% preischemic values, respectively. In contrast, coronary flow was 82 ± 19%, 79 ± 15%, and 102 ± 30%

Table 1. Baseline characteristics.

	Ischemic group				
	37° C/20 min	37° C/30 min	32° C/40 min	32° C/50 min	32° C/60 min
N	5	5	6	6	6
Heart weight (g)	2.0 ± 0.2	1.8 ± 0.2	1.9 ± 0.1	2.2 ± 0.4	2.1 ± 0.2
Heart rate (beats/min)	248 ± 18	248 ± 12	252 ± 20	237 ± 18	239 ± 11
PSP (mmHg)	88.7 ± 5.6	86.6 ± 4.9	85.4 ± 2.8	94.6 ± 2.4*	94.3 ± 2.9*
RPP (mmHg·beats/min × 10^{-3})	21.9 ± 2.4	21.4 ± 1.4	21.5 ± 1.4	22.8 ± 0.7	22.3 ± 1.1
Cardiac output (ml/min)	50.3 ± 5.5	42.6 ± 13.2	41.9 ± 2.9#	58.8 ± 3.4	58.1 ± 3.4
Coronary flow (ml/min)	23.8 ± 5.0	21.3 ± 6.7	22.5 ± 6.1	24.3 ± 2.6	25.1 ± 1.8
O_2C (mmHg·ml/min·g/wet)	3760 ± 767	3598 ± 1286	3525 ± 582	3333 ± 696	3506 ± 644

All parameters other than heart and body weight were calculated as the average of the 10-, 20-, and 30-min preischemic values for each heart.
O_2C, oxygen consumption; PSP, peak systolic pressure; RPP, (PSP*heart rate).
*$P < 0.05$ vs. 37° C/30 min and 32° C/40 min; #$P < 0.01$ vs. 32° C/50 min and 32° C/60 min.

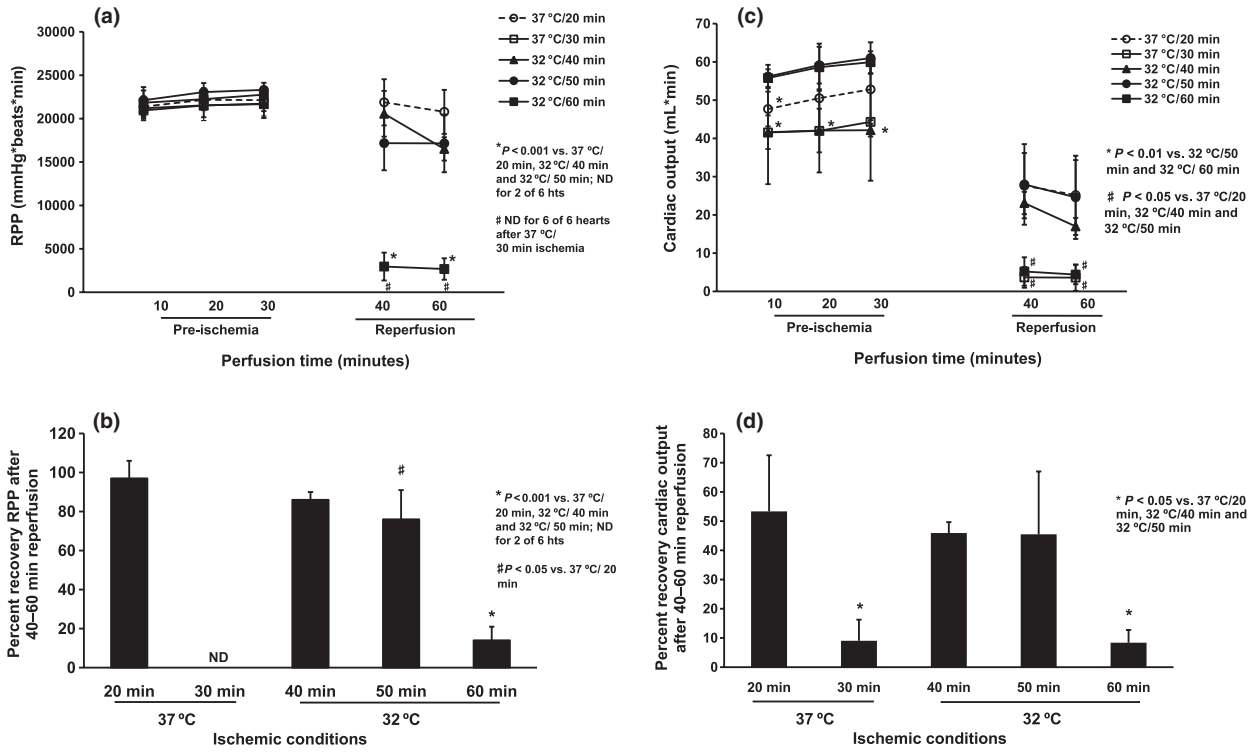

Figure 2 Contractile function: RPP (heart rate–peak systolic pressure product) and cardiac output. Absolute values of heart rate–peak systolic pressure product (RPP) are reported for preischemic and loaded reperfusion periods in panel (a). Percent recovery RPP after 40- to 60-min reperfusion of preischemic values are presented in panel (b). RPP was not detectable (ND) during reperfusion in two of six hearts in the 32° C/60 min ischemic group, or in any heart exposed to 37° C/30 min ischemia. Absolute values and percent recovery after 40- to 60-min reperfusion for cardiac output are presented in panels (c) and (d), respectively.

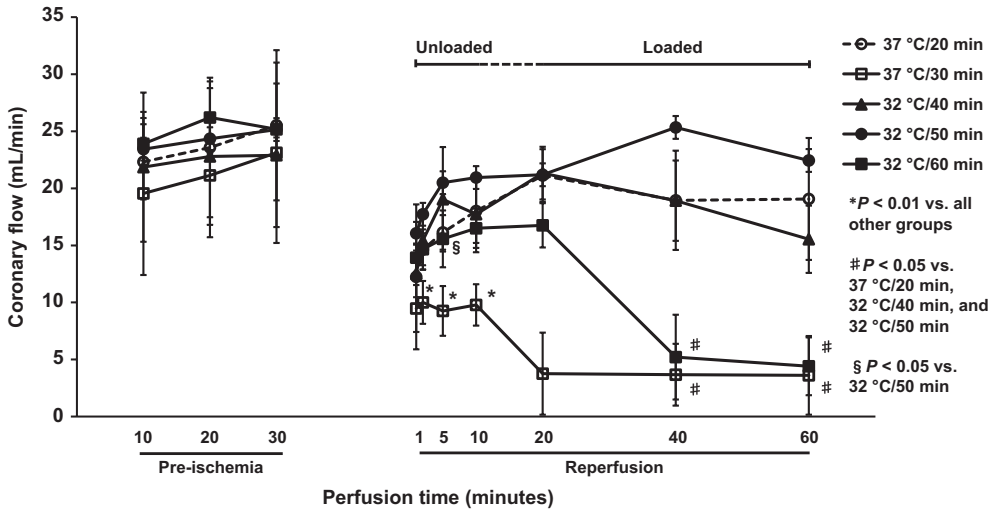

Figure 3 Coronary flow: absolute values of coronary flow are reported for the preischemic and entire reperfusion periods. Importantly, hearts were perfused for the first 10–20 min of reperfusion in an unloaded, retrograde mode with a perfusion pressure of 60 mmHg. Statistical comparisons were made between ischemic groups at 10, 40, and 60 min of reperfusion.

preischemic values in, respectively, 37° C/20 min, 32° C/40 min, and 32° C/50 min ischemic groups (hearts that recovered). Accordingly, coronary flow was significantly greater at 40- and 60-min reperfusion time points in hearts that recovered compared with those that did not ($P < 0.05$ for all comparisons).

Percentage recovery of coronary flow was also determined at 10-min reperfusion (Fig. 4). At this time point, recovery of coronary flow relative to preischemic values was >75% among hearts that recovered, and generally lower in groups that did not recover (significantly lower for 37° C/30 min (49 ± 14%) vs. 32° C/40 min (82 ± 17%) and 32° C/50 min (88 ± 9%) and for 32° C/60 min (66 ± 10%) vs. 32° C/50 min (88 ± 9%); $P < 0.05$ for all comparisons).

As stated above, in hearts that did not recover (37° C/30 min or 32° C/60 min ischemic groups), coronary flow tended to be lower during early reperfusion (Figs 3 and 4). These hearts were relatively underperfused during reperfusion and data for parameters dependent upon coronary flow must thus be interpreted with care. As such, we chose to report values for lactate, troponin T, and lactate dehydrogenase accumulation, as well as oxygen consumption at 10 min of reperfusion when all hearts were still in unloaded mode and exposed to the same perfusion (afterload) pressure. Therefore, our values for these parameters may have underestimated the production of lactate, Troponin T (TnT), and LDH; we consider these values to be minimum estimations.

Effects of mild hypothermia during ischemia on metabolic recovery

During the preischemic period of perfusion, lactate was not detectable in coronary effluent or circulating buffer samples (data not shown). Over the initial 10-min unloaded period of reperfusion, during which the coronary flow was most similar among all groups, lactate accumulation in perfusate was not detectable in any heart exposed to ischemia at 37° C for 20 min or at 32° C for 40 min, whereas it was measurable in all other groups above the approximate detection limit of 20 μmol/g wet weight (Fig. 5a).

During preischemic perfusion, oxygen consumption was similar among groups (Table 1). At 10-min reperfusion, recovery of oxygen consumption was higher in 32° C/40 min, 32° C/50 min, and 32° C/60 min ischemic groups vs. 37° C/30 min group and in the 32° C/50 min ischemic group vs. the 32° C/40 min ischemic group, ($P < 0.05$ for all comparisons; Fig. 5b).

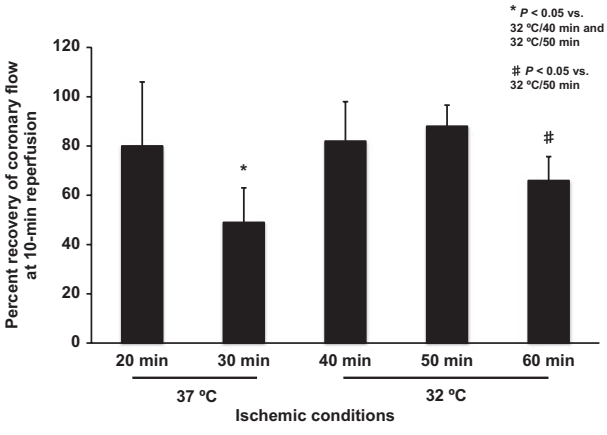

Figure 4 Coronary flow: percent recovery after 10-min reperfusion. Percent recovery coronary flow of preischemic values after 10-min reperfusion are presented.

Effects of mild hypothermia during ischemia on cardiac necrosis

Markers of necrosis were generally elevated in hearts that did not recover (37° C/30 min or 32° C/60 min ischemic groups), compared with those recovering well (Fig. 6a and b). TnT accumulation in perfusate after 10-min reperfusion was significantly greater in 37° C/30 min, 32° C/50 min, or 32° C/60 min ischemic groups vs. 37° C/20 min, 32° C/

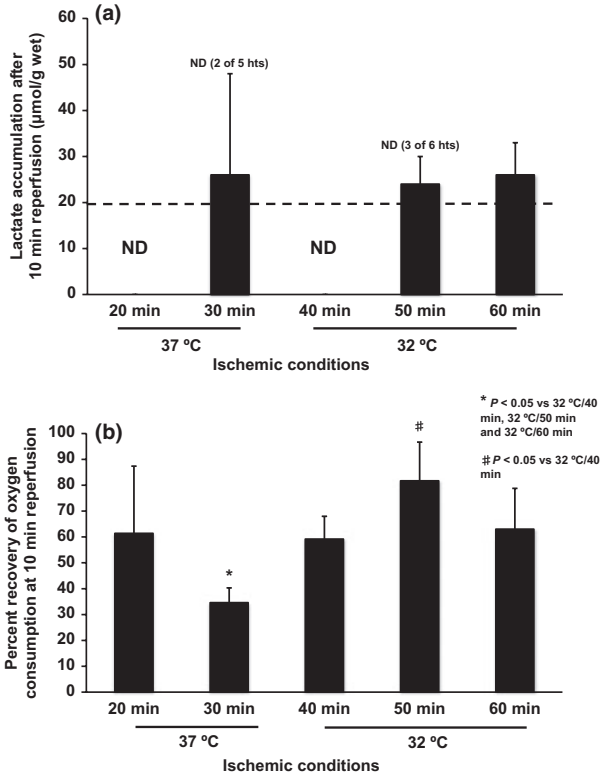

Figure 5 Markers of metabolism: lactate production and oxygen consumption. Total absolute values of lactate were measured in recirculating buffer after 10 min of reperfusion (a). Approximate threshold limit of detection for lactate accumulation is represented by dashed line. Lactate was not detectable (ND) during reperfusion in the 37° C/20 min ischemic group, nor in the 32° C/40 min ischemic group. Percentage recovery oxygen consumption at 10-min reperfusion is presented in panel (b).

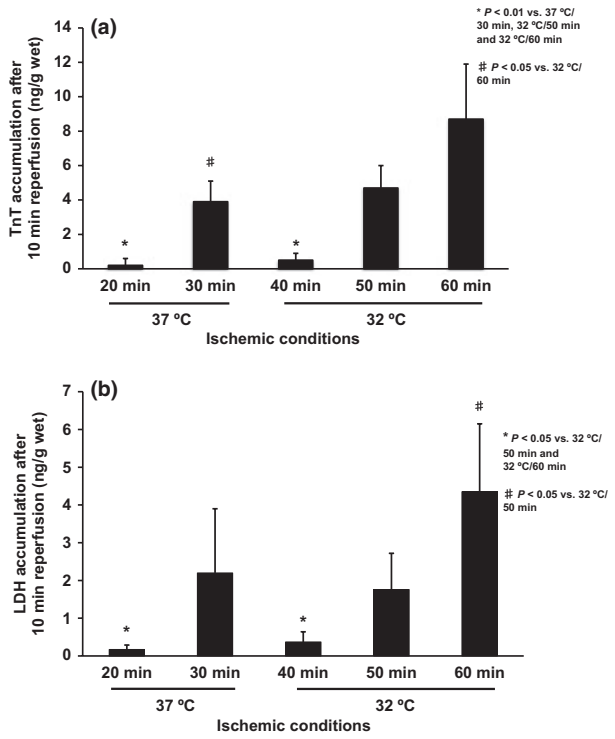

Figure 6 Markers of necrosis: Troponin T (TnT) and lactate dehydrogenase (LDH). Total absolute values of TnT (a) and LDH (b) were measured in recirculating buffer after 10 min of reperfusion.

40 min groups ($P < 0.01$ for all comparisons, respectively). Similarly, LDH accumulation in perfusate after 10-min reperfusion was significantly greater in 32° C/50 min or 32° C/60 min ischemic groups vs. 37° C/20 min, 32° C/40 min groups ($P < 0.05$ for all comparisons).

Discussion

In this study, we have demonstrated that simple reduction in cardiac temperature by a few degrees rapidly after the onset of global ischemia dramatically prolongs the interval during which the heart remains resistant to functional and structural deterioration. In this sense, our results provide evidence for a larger window of ischemic tolerance than previously recognized for DCD hearts. Indeed, in previous studies investigating transplantation with DCD hearts not using ischemic pretreatments (except for heparin according to clinically acceptable procedures for DCD), good recovery of contractile function has generally been limited to hearts exposed to a period of warm ischemia of ≤ 30 min [9–12]. Importantly, we demonstrate that postischemic induction of mild hypothermia allows preservation of contractile capacity for an ischemic period that is approximately twofold longer than that observed at 37° C; thereby providing a first, but mandatory, step toward optimal and legally/ethically acceptable preservation of DCD cardiac grafts. These findings should inspire further fundamental and clinical research, and potentially lead to greater recognition of DCD in heart transplantation.

Hypothermia is unanimously recognized as cardioprotective when applied at the time of cardiac arrest. Consequently, hypothermia is generally considered as a critical component of cardioplegic strategies. In the vast majority of cardiac ischemic tolerance studies, protocols involve hearts that were cooled and/or cardiopleged before the initiation of ischemia. In contrast, this study aimed to simulate the clinical situation of a DCD in an isolated working rat heart preparation; therefore, hearts were exposed to global ischemia before mild hypothermia was applied, that is, hearts were not arrested with hypothermia or under hypothermic conditions. Thus, unlike most studies, this study incorporates a period of global ischemia, which subsequently provokes cardiac arrest. This represents a critical difference, compared with most previous studies, as warm ischemia is more damaging than hypothermic and/or cardioplegic ischemia. Furthermore, this period of cardiac ischemia is of particular concern in DCD, as protective strategies are not currently implemented prior to its onset. We observed that reducing ischemic temperature to 32° C after ischemic onset doubles myocardial ischemic tolerance in terms of contractile recovery. Our findings in hearts exposed to 40 min of mildly hypothermic ischemia suggest that this cardioprotective effect may involve better coronary perfusion, improved metabolic recovery, and less necrosis upon reperfusion.

Locally applied and noninvasively induced mild hypothermia may be one of the very few preservation means that is ethically applicable in the context of DCD. With DCD, interventions are limited for ethical reasons, and no direct access to the heart is permitted immediately following cardiac arrest. Thus, although benefits of mild hypothermia likely depend on its rapid application, cardioprotective protocols targeting the warm ischemic period must respect limitations surrounding DCD to be clinically acceptable. Furthermore, no cardioprotective temperature threshold has been clearly defined [13]; previous reports have shown that even a minor temperature reduction (35° C) provides a protective effect [16], suggesting that even lowering cardiac temperature by a couple of degrees would be beneficial. In our experimental model, we have chosen to use a target temperature of 32° C, as at this temperature, the heart continues to beat and is able to support systemic circulation [13].

We speculate that our results could be integrated into the current context of DCD in two ways. First, mild cardiac hypothermia could potentially be commenced prior to cardiac arrest in the clinical setting of DCD when donor status has been confirmed and withdrawal of treatment is

planned. We hypothesize that a noninvasive process to cool the myocardium, such as a cooling thoracic jacket combined with insufflation of cold air into the lungs, might be sufficient to reduce heart temperature by a few degrees. In fact, strategies for cardiac cooling have been demonstrated to be clinically feasible [17]. The EMCOOLS System, for example, is indicated in patients with an acute infarct and is composed of external pads that are placed around the major part of the body. Although patients were not in circulatory arrest, this system decreased the temperature of the heart by 1.1° C/h [18]. It could thus be expected that heart temperature would decrease more rapidly in a patient with circulatory arrest. In this particular DCD setting (planned withdrawal of treatment), currently available cooling techniques could effectively reduce cardiac temperature by a few degrees prior to the onset of cardiac ischemia. Secondly, given that multiple methods to rapidly induce mild hypothermia are currently being developed and tested [13, 14, 19], our results highlight the potential need for rapid cooling techniques in the context of DCD.

Mechanisms responsible for cardioprotective effects of mild hypothermia are not completely understood, but several processes are likely involved [20]. Anti-infarct effects may result, at least in part, through limiting vascular damage and subsequent microvascular obstruction, thereby improving coronary perfusion [21, 22] and reduction in no-reflow phenomenon [13, 14]. Our findings essentially confirm this assumption, as compared with hearts that underwent ischemia at 37° C for 20 min; those that underwent ischemia at 32° C demonstrated similar coronary flows, despite extension of ischemia to up to 50 min. However, the precise cause(s) of reduced coronary flow in the 37° C/30 min and 32° C/60 min ischemic groups remain to be determined; postischemic reductions in coronary flow under these conditions will be addressed in future studies.

In general, hypothermia is believed to act by lowering the rates of cellular reactions that contribute to ischemic injury [7]. Cardioplegic preservation for instance typically involves rapidly arresting and cooling the heart to reduce metabolic demand, thereby preserving ATP for essential cellular functions. However, unlike profound hypothermia, mild hypothermia is believed to have only a modest effect on cardiac energy depletion during ischemia [23]. These findings have led to the speculation that mild hypothermia during ischemia likely involves cardioprotective mechanisms in addition to the effects on energy metabolism [24]. Lopaschuck *et al.* have proposed that the imbalance between glycolysis and glucose oxidation plays a critical role in cardiac ischemia-reperfusion injury [25]. During a period of severe no-flow ischemia, anaerobic metabolism is favored, such that rates of glycolysis are accelerated relative to glucose oxidation; lactate and H^+ ions accumulate, and can lead to increased Ca^{2+} overload during subsequent reperfusion [26, 27]. Importantly, reduction in the mismatch between glycolysis and glucose oxidation improves contractile recovery upon reperfusion [25]. In this study, we found a reduction in lactate production in the early period of reperfusion following ischemia at 32° C, consistent with the concept that mild hypothermia during ischemia is associated with a reduced mismatch in glucose metabolism upon reperfusion.

Although oxygen consumption is generally considered to correlate positively with postischemic recovery, it is critical to consider coronary flow and contractile work in parallel. We report here that oxygen consumption is similar among groups that recovered contractile function even to a small degree (37° C/20 min, 32° C/40 min, 32° C/50 min, and 32° C/60 min ischemic groups); while oxygen consumption was significantly lower in hearts that showed no signs of functional recovery (37° C/30 min ischemic group). However, in this latter group, coronary flow was also significantly lower; thus, we cannot distinguish between truly lower tissue oxygen consumption and reduced coronary perfusion leading to low oxygen consumption. Nevertheless and interestingly, oxygen consumption at 10-min reperfusion was similar among groups, which subsequently recovered >80% RPP (37° C/20 min and 32° C/40 min), indicating that mild hypothermia during ischemia permits similar metabolic recovery despite a twofold longer period of ischemia.

Several cellular signaling pathways have been recently implicated in reduction in ischemia-reperfusion injury induced by mild hypothermia (reviewed in [14]), and likely involve inhibition of Ca^{2+}-induced MPT (mitochondrial permeability transition) [28], which is, at least in part, involved in the ischemic protection afforded by pre- and postconditioning [29, 30]. Importantly, inhibition of MPT occurs during the first minutes of reperfusion only if the heart has been injured ('primed') during the ischemic period [30, 31]. Thus, it has been proposed that mild hypothermia during ischemia may inhibit MPT through the reduction in the 'priming' effect [13]. Hearts in this study were reperfused with buffer at 37° C; thus providing evidence that beneficial effects of mild hypothermia involve a mechanism that occurs, or is triggered, during the ischemic period itself. These aspects deserve further evaluation in this setting.

In this study, hearts were perfused with a high, physiologically relevant, levels of fatty acid (1.2 mM palmitate). We chose to do so as elevated levels of circulating fatty acid occur during most cardiovascular surgery interventions [32] and are detrimental to recovery of postischemic contractile function. High circulating levels of fatty acids drive fatty acid oxidation, thereby inhibiting glucose oxidation, leading to greater mismatch between glycolysis and glucose

oxidation, which may result in greater acidosis and calcium overload [25, 33]. As such, this study employed conditions that were not necessarily the most favorable for heart recovery, but that were of physiologic relevance. Taken together, high fatty acids levels are of particular importance in the setting of heart transplantation with DCD and our findings provide evidence that mild hypothermia during ischemia can dramatically prolong ischemic tolerance despite the presence of high, physiologically relevant, levels of fatty acids.

Our protocol obviously has some limitations. Our studies included perfusion with KH solution and thus may not completely reflect a clinical situation with blood. Furthermore, only acute effects of mild hypothermia during ischemia were investigated in this study. Additional investigations are required to determine the effects of mild hypothermia during cardiac ischemia on recovery at sub-acute and chronic time points.

Conclusion

We demonstrate that inducing a localized mild hypothermia is sufficient to dramatically prolong ischemic tolerance of nonoxygenated hearts. The simplicity of this approach might thus open a window of opportunity to facilitate the development of a clinical approach to include, in the near future, DCD in heart transplantation programs. Indeed, as warm ischemia occurs prior to heart procurement with DCD, but not with conventional, brain-dead donors, means to reduce ischemia-reperfusion injury in this setting has received little research attention. As clinical protocols for procurement of DCD organs, including the heart, are in the process of being established, we believe that our proposed work has potential to aid in the establishment of heart procurement protocols in this context. Further investigations are nevertheless required, on one hand, to provide greater insight into the mechanisms of ischemia-reperfusion injury and its prevention, especially in the setting of DCD; and, on the other hand, to confirm these first observations in more clinically relevant models.

Authorship

MS and MD: involved in performing experiments, analysing data and manuscript writing. DC: involved in performing experiments and analysing data. BG: involved in analysing data, performing statistical analyses and manuscript writing. FD: involved in manuscript writing. TC: involved in study design and manuscript writing. HT: involved in study design, data analysis and manuscript writing. SL: involved in study design, performing experiments, data analysis and manuscript writing.

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