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Article type : Original Article

Cardioprotective reperfusion strategies differentially affect mitochondria: studies in an isolated rat heart model of donation after circulatory death (DCD)

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/ajt.15024

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List of abbreviations:

AK, adenylate kinase; ATP, adenosine triphosphate; AU: arbitrary units; CF, coronary flow; CI, mitochondrial complex I; CII, mitochondrial complex II; CK, creatine kinase; CO, cardiac output; COX, cytochrome c oxidase (mitochondrial complex IV); CS, citrate synthase; cyt c, cytochrome c; DBD, donation after brain death; DCD, donation after circulatory death; DP, developed pressure; dP/dt_{max}, maximum first derivative of left ventricular pressure; dP/dt_{min}, minimum first derivative of left ventricular pressure; HR, heart rate; HY, hypoxia; IR, ischemia and reperfusion; IU, international unit; KHB, Krebs-Henseleit bicarbonate buffer; LDH, lactate dehydrogenase; LV, left ventricular; MH, mild hypothermia; MPC, mechanical post-conditioning; min, minute(s); mtDNA, mitochondrial DNA; mtDNA:nDNA, mitochondrial DNA-nuclear DNA ratio; mtPTP, mitochondrial permeability transition pore; nDNA, nuclear DNA; N.D, non detectable; O_2, oxygen; O_2C, oxygen consumption; O_2:T, oxygen consumption-triple product ratio; PCr, phosphocreatine; ROS, reactive oxygen species; sec, second(s); SD, standard deviation; TBARS, thiobarbituric acid reactive substances; TnT, troponin-T; vs., versus; wt, weight

Abstract

Donation after circulatory death (DCD) holds great promise for improving cardiac graft availability, however concerns persist regarding injury following warm ischemia, after donor circulatory arrest, and subsequent reperfusion. Application of pre-ischemic treatments is limited for ethical reasons, thus cardioprotective strategies applied at graft procurement (reperfusion) are of particular importance in optimizing graft quality. Given the key role of mitochondria in cardiac ischemia-reperfusion injury, we hypothesize that three reperfusion strategies: mild hypothermia, mechanical post-conditioning and hypoxia, when briefly applied at reperfusion onset, provoke mitochondrial changes that may underlie their cardioprotective effects. Using an isolated, working rat heart model of DCD, we demonstrate that all three strategies improve oxygen-consumption–cardiac-work coupling and increase tissue ATP content, in parallel with increased functional recovery. These reperfusion strategies, however, differentially affect mitochondria; mild hypothermia also increases phosphocreatine content, while mechanical post-conditioning stimulates mitochondrial complex I activity and reduces cytochrome c release (marker of mitochondrial damage), whereas hypoxia up-regulates the expression of Pgc-1α (regulator of mitochondrial biogenesis). Characterisation of the role of mitochondria in cardioprotective reperfusion strategies should aid in the identification of new, mitochochondrial-based therapeutic targets and the development of effective reperfusion strategies that could ultimately facilitate DCD heart transplantation.

1. Introduction

Heart transplantation is currently the best option to improve quality of life and life expectancy of patients suffering from end-stage heart failure. However, the number of donors is largely insufficient to cover the ever-increasing demand for hearts in both the EU

and the US (1,2). One promising solution to this recurrent shortage is the use of grafts obtained from donors after circulatory death (DCD) (3).

Despite ground-breaking demonstrations of clinical DCD heart transplantation in the last four years (4–7), concerns about graft quality persist. Indeed, DCD hearts are inevitably exposed to warm, global ischemia between circulatory arrest and procurement that can rapidly lead to tissue damage. Nonetheless, donor hearts can withstand a short period of warm ischemia; its duration has been limited to 30 min in recent DCD heart transplantations (8).

As interventions in DCD donors are limited for ethical reasons, cardioprotective therapies applied immediately following the period of warm ischemia hold great promise to increase graft tolerance to ischemia-reperfusion-induced injury (9–12). In current clinical practice, cardiac grafts are reperfused immediately following warm ischemia with either cold cardioplegia supplemented with erythropoietin and glyceryl nitrate with (4) or without (5) heparin (direct procurement protocol), or they are reperfused *in situ*, following heparinization, with donor blood (normothermic regional perfusion protocol) (6). Therefore, differing approaches are used for reperfusion following warm ischemia in the clinical setting, and it is likely that further development of alternative and/or complementary reperfusion strategies will contribute towards the optimization of graft quality (8).

Mitochondria play an important role in cardiac ischemia-reperfusion (IR) injury. During cardiac ischemia, reliance on glycolysis leads to intracellular acidosis and Ca^{2+} overload, which contribute to the progressive deterioration of mitochondrial structure and function (13). During reperfusion, recovery of mitochondrial oxidative phosphorylation for ATP generation is a critical requirement for recovery of cardiac function. However, mitochondria themselves contribute to cellular damage at reperfusion, through the exacerbated production of reactive oxygen species (ROS). ROS damage cellular membranes and increase the sensitivity of mitochondrial permeability transition pore (mtPTP) to Ca^{2+} (13). With the normalization of intracellular pH at the onset of reperfusion, these processes lead to the opening of mtPTP,

which can trigger mitochondrial destruction and cell death (13). For these reasons, preservation of mitochondrial function and integrity during the first minutes of reperfusion is of central importance for optimizing recovery of hearts exposed to warm ischemia (14,15).

We have previously described three physical strategies as cardioprotective: mild hypothermia (MH), mechanical postconditioning (MPC), and hypoxia (HY); when applied during the first minutes of reperfusion following global, warm ischemia (9). Interestingly, MH has been reported to prevent mtPTP opening when applied at reperfusion for 1 hour or 30 min in both, *in vivo* or *in vitro* models of IR, respectively (16,17). In addition, MPC has been linked with better preserved mitochondrial function, including limited ROS production and delayed mtPTP opening (10,18–21). Furthermore, although HY would be expected to protect mitochondria by reducing or slowing the ROS burst at reperfusion, its antioxidative effect is still under debate (22,23). In summary, MH, MPC and HY all demonstrate cardioprotective effects and although underlying mechanisms remain incompletely understood, evidence supports roles for cardiac mitochondria.

Therefore, we hypothesize that all three physical cardioprotective strategies: MH, MPC and HY, provoke mitochondrial changes which may underlie their cardioprotection effects. In this study, we report our investigation into mitochondrial changes induced by the application of these strategies in an isolated rat heart model of DCD.

2. Materials and methods

2.1. Animals and ethical statement. Male Wistar rats obtained from Janvier Labs (Le Genest-Saint-Isle, France) were housed under standard conditions with unrestricted access to food and water. Animal handling was in compliance with the European legislation (86/609/EEC) and the Swiss Animal Protection Ordinance.

2.2. Study design. An isolated, perfused heart was used as a model of DCD. Importantly, this model was used to simulate the period of warm, global ischemia, to which DCD organs are exposed. In our experimental model, hearts were perfused *ex situ* prior to ischemia, and cardioprotective strategies were applied using a warm (30 or 37°C), physiologic crystalloid buffer perfusate in order to precisely control the conditions to which the hearts are exposed and to limit potentially negative effects in donor blood or cold cardioplegia. Furthermore, directly after the application of cardioprotective strategies in an initial period of unloaded reperfusion, we proceeded with a loaded perfusion period to assess functional recovery in order to limit the effects of potentially confounding factors that may occur with additional cardioplegic/storage periods.

Prior to anesthesia, hearts were randomly allocated to one of five parallel-arm, experimental groups: 1) control (non-treated ischemic group); 2-4) MH, MPC or HY (treated ischemic groups); and 5) sham (non-ischemic control group; Figure 1).

2.3. Isolated, working heart preparation. Hearts were procured and initially perfused in Langendorff-mode with modified Krebs-Henseleit bicarbonate buffer (KHB) within 2 min of diaphragm transection (9). The left atrium was then cannulated, and the perfusion switched to loaded (working) mode (24).

2.4. Perfusion protocol. All hearts were initially submitted to 20 min of aerobic, working baseline perfusion (afterload 80 mmHg) with modified KHB (9). During this period, the perfusate was supplemented with 1.2 mM palmitate and 3% bovine serum albumin to simulate high pre-ischemic circulating fatty acid levels, as expected in clinical DCD (25). Unless otherwise stated, perfusion buffers were oxygenated with 95% O_2/5% CO_2 to achieve a pO_2 of 550 mmHg, and maintained at 37°C.

Control, MH, MPC and HY groups then all underwent 27 min warm (37°C), global ischemia; perfusate lines were clamped and hearts were immersed in energy-substrate-free KHB bubbled with 95% N_2/5% CO_2. Hearts in the sham group were maintained under baseline perfusion conditions for the equivalent ischemic duration.

All groups underwent reperfusion for 60 min with modified KHB (no palmitate supplementation). Reperfusion was initiated in unloaded mode for the first 10 min and then switched to loaded mode for the remaining 50 min (afterload 60 mmHg). Afterload pressure was set to 60 mmHg during reperfusion to ensure sufficient perfusion and limit the risk of exacerbated post-ischemic damage (26). Reperfusion strategies were applied as previously described (9): MH, perfusate at 30°C for the first 10 min of reperfusion; MPC, 2 cycles of 30 sec reperfusion and 30 sec ischemia; and HY, perfusate bubbled with 95% N_2/5% CO_2 for the first 2 min reperfusion.

Detailed methods for the measurements of contractile cardiac function, biochemical and molecular parameters can be found in Supplemental materials.

3. Results

3.1. Baseline characteristics. Absolute values for perfusion parameters measured at 20 min of baseline, as well as rat body and heart weights, are presented in Table S1. A total of 39 rats were included: sham (n=8); control (n=8); MH (n=8); MPC (n=8); HY (n=7). All parameters were similar between the ischemic non-treated (control) group and reperfusion-strategy groups, except for a higher body weight in the HY group ($p < 0.05$). Baseline values measured for the sham group tended, however, to differ from other groups and some, essentially CO, reached statistical significance.

3.2. Post-ischemic recovery. Cardiac recovery is presented as percentage (60 min reperfusion value expressed as a percentage of mean pre-ischemic value; Figure 2A-H). Percentage recovery was significantly reduced in control compared to sham hearts for all parameters. All reperfusion strategies significantly improved LV work compared to control hearts ($p < 0.05$ HY; $p < 0.001$ MH and MPC; Figure 2A). Most other parameters were significantly improved with reperfusion strategies compared with control hearts, although the profile of significant differences varied among strategies (Figure 2B-H). No difference among

groups was observed for recovery of CF (data not shown). Absolute values for these parameters at 60 min reperfusion are shown in Table S2.

3.3. Oxygen metabolism. Oxygen consumption (O_2C) was significantly decreased at 20, 40, and 60 min reperfusion in control versus sham hearts ($p < 0.05$ for all), and significantly increased with MPC compared to controls at 40 and 60 min reperfusion ($p < 0.05$ at 40 min; $p < 0.01$ at 60 min; Figure 3A). Oxygen consumption-triple product ratio (O_2:T), a marker of the coupling between cardiac oxygen consumption and the work performed, with a higher value indicating a greater uncoupling, is shown in Figure 3B. O_2:T was significantly increased in control vs. sham at 20 and 40 min reperfusion ($p < 0.05$ for both) and then decreased to sham values at 60 min reperfusion. All three strategies prevented the ischemia-induced increase and the corresponding values were significantly lower than control values at 40 min reperfusion ($p < 0.05$ all vs. control). Absolute values for these parameters at 60 min reperfusion are shown in Table S2.

3.4. Release of markers of cellular death and mitochondrial damage during reperfusion. Release of troponin-T (TnT) and cytochrome c (cyt c), as well as lactate accumulation, were significantly increased in control versus sham hearts at all time points ($p < 0.05$ for all; Figure 4A, C, D). LDH was not detectable (N.D) in sham hearts (Figure 4B). MPC was the only strategy to significantly modify these markers compared to control hearts; specifically, MPC increased TnT release at 10 min reperfusion ($p < 0.05$, Figure 4A) and decreased the release of cyt c at 40 min reperfusion ($p < 0.05$, Figure 4C).

3.5. Myocardial energy status. ATP content measured at 60 min reperfusion was reduced by 62% in the control compared to the sham group ($p < 0.001$; Figure 5A). Interestingly, all reperfusion strategies partially restored ATP levels ($p < 0.05$ MPC and HY vs. control; $p < 0.001$ MH vs. control). PCr content was also reduced in the control compared to sham group ($p < 0.05$), but to a lesser extent than ATP (25% reduction; Figure 5B), and MH restored PCr content ($p < 0.05$ MH vs. control) to values comparable to those in sham hearts. In parallel with ATP, ADP content was reduced by 44% in the control compared to the sham

group ($p < 0.05$; Figure 6C). ADP content was also reduced in all reperfusion strategy groups vs. sham ($p < 0.01$ for all), and appeared to be slightly lower than that in the control group, although not statistically different. Consequently, the ATP/ADP ratio was almost identical in ischemic non-treated (control) and sham hearts, and it tended to be slightly increased, although not statistically significant, in all reperfusion strategy groups (Figure 5 D).

3.6. Mitochondrial-related enzymes activity. Of all mitochondrial-related enzyme activities, only mitochondrial complex I (CI) was significantly decreased (25%) in control vs. sham groups ($p < 0.05$; Figure 6A). MPC was the sole strategy that increased mitochondrial CI activity compared with controls ($p < 0.05$). No significant difference among groups was observed for mitochondrial complex II (CII) (Figure 6B) or adenylate kinase (AK) (Figure 6C). Although creatine kinase (CK) activity was not modified in control vs. sham groups (Figure 6D), it was significantly increased in HY compared to sham, MH and MPC hearts ($p < 0.01$ sham vs. HY; $p < 0.05$ MH and MPC vs. HY).

3.7. Mitochondrial content. No significant difference was observed between control and sham groups for citrate synthase (CS) activity (Figure 7A), nor for cytochrome c oxidase (COX) activity (Figure 7B). In contrast, the mtDNA:nDNA (mitochondrial DNA-nuclear DNA ratio) was significantly increased in control compared to sham hearts ($p < 0.001$; Figure 7C). Reperfusion strategies did not modify any of these parameters compared to control values.

3.8. mRNA expression of genes involved in mitochondrial biogenesis. 27 min ischemia followed by 60 min reperfusion did not significantly modify the mRNA expression of genes involved in the mitochondrial biogenesis pathway (Figure 8 A-E). HY was the sole strategy to significantly elevate the expression of PCG-1α, a master regulator of mitochondrial biogenesis, compared to the control group ($p < 0.05$; Figure 8D).

3.9. Markers of oxidative stress. Cardiac TBARS content was similar among experimental groups (Figure 9A), while protein carbonylation was significantly increased in control vs. sham hearts ($p < 0.01$; Figure 9B). Reperfusion strategies did not modify either of these parameters compared to control values.

3.10. Correlations between mitochondrial, metabolic, and contractile parameters. Correlation analyses were performed to investigate relationships between cardiac recovery at 60 min reperfusion and either, i) mitochondrial-related parameters at 60 min reperfusion (Figure 10), or ii) mitochondrial- and cell death- related parameters at early (10 min) reperfusion (Figure 11). Significant correlations in both analyses revealed differing profiles among strategies.

4. Discussion

Using our isolated, working rat heart model of DCD, we report that three, briefly applied reperfusion strategies, MH, MPC, and HY, improve the post-ischemic cardiac functional recovery in association with modification of mitochondrial-related parameters. Although these strategies induced differing effects on mitochondria, all provoked a greater recovery of ATP stores and quicker normalization of oxygen consumption-cardiac work coupling compared to untreated controls. To our knowledge, this is the first demonstration that a reduction in temperature of a few degrees, applied as a cardioprotective strategy alone for only the first few minutes of reperfusion (MH), leads to increased tissue content of ATP and PCr one hour later. We also report for the first time that with MPC, mitochondrial CI activity is increased in parallel with tissue ATP content and reduced mitochondrial damage. Likewise, our study provides the first demonstration that short application of HY at reperfusion onset preserves cardiac contractile function and improves ATP content subsequently. A more comprehensive understanding of the mechanisms of action underlying cardioprotection provided by the reperfusion strategies should help in the development of new therapeutic tools, which may ultimately be used to promote optimal recovery of graft function in DCD hearts.

In pre-clinical models of DCD, cardioprotective effects of MH have been previously reported when applied for longer periods during reperfusion (1-3 hours) (27,28), or when applied in combination with other cardioprotective conditions/agents during the first minutes

of reperfusion (29). However, our study is the first to investigate MH as a sole strategy when applied for a brief period at the onset of reperfusion. We demonstrate that MH induces preservation of cardiac contractile function in association with greater replenishment of high energy metabolites (ATP and PCr). This finding is in agreement with that of Tolboom, also in a rat model of DCD, in which MH applied for 1 hour reperfusion led to increased repletion of ATP levels compared to non-treated ischemic hearts (27). Therefore, although various MH treatments have been demonstrated to increase mitochondrial respiration (16), delay mtPTP opening (16) or reduce cellular oxidative stress (27), our results reveal that only a very short application of MH (10 mins) at the beginning of reperfusion is required to induce a significant improvement in repletion of high-energy metabolites over the first hour of reperfusion.

We also report that MPC preserves post-ischemic cardiac function, in association with increased ATP levels and mitochondrial CI activity. MPC is a reperfusion strategy that has been widely studied (10,18–21,30–32), although its precise mechanism of action remains to be elucidated. Our observation of MPC-induced increases in cardiac ATP content agrees with previous studies (19,20,30), and it is tempting to speculate that increased mitochondrial CI activity contributes to these higher ATP levels. However, previous studies have demonstrated varying effects of MPC on mitochondrial CI activity (10,18,31). It should be noted that mitochondrial CI is of particular importance in IR injury as it is the main cellular producer of ROS (33). As such, our finding of higher mitochondrial CI activity could be associated with higher ATP synthesis, but as well, it could also lead to excessive ROS production. Importantly, in MPC hearts compared with controls, cyt c release, a recognized indicator of mitochondrial damage (34) was significantly reduced at 40 min reperfusion and oxidative stress at 60 min reperfusion was not increased (although we cannot exclude that greater oxidative stress was induced by MPC at earlier reperfusion timepoints). Thus, our findings support the concept that MPC-induced mitochondrial CI activation and preserved mitochondrial integrity, which may contribute to greater ATP content and contractile recovery.

To our knowledge, we are the first to reveal that short hypoxic conditions at reperfusion onset (HY), promote post-ischemic recovery of cardiac contractile function in parallel with higher ATP content. HY was aimed to reduce the burst of mitochondrial ROS production that accompanies oxygen re-introduction at reperfusion. However, others have reported that similar strategies provoke either cardioprotection and antioxidant effects (22,35), or a lack of protection in combination with heightened oxidative stress (23). We did not observe HY-induced reductions in overall (net) oxidative stress; however, our investigations do not provide insight as to whether ROS production and/or antioxidant capacity in these hearts was altered by HY – further investigation is required to answer these specific questions. Strikingly, we demonstrate for the first time that HY increases CK activity compared to most other experimental conditions. This is of particular interest in light of recent reports that *in vitro* (36) or *ex vivo* (37) overexpression of specific CK isoforms is cardioprotective. Thus, our novel demonstration of HY-induced CK activation merits futher investigation to determine its importance in cardioprotection, as well as the underlying mechanisms.

The application of reperfusion strategies stimulated ATP repletion during reperfusion. We report a substantial decrease in tissue ATP content after 27 min warm, global ischemia and 60 min reperfusion; a 62% reduction vs. non-ischemic values. This marked decrease contrasts with studies using unloaded (Langendorff) heart perfusions (19,20,27,30). In our study, the maximum reperfusion-strategy-induced repletion reached approximately 50% non-ischemic values, whereas in “unloaded” models, similar strategies fully restored the ATP content (19,20,27,30). It is unclear why we observe lower post-ischemic ATP levels, but it may be due to greater ATP demand as a consequence of our experimental conditions: tight ischemic temperature control (36.5°C-37.2°C) and a loaded heart preparation (38). We also report a drop in tissue ADP content after IR, similar to other animal models (39,40). However, reperfusion strategies tended to further decrease ADP levels, leading to a trend towards higher ATP/ADP ratios in reperfusion-strategy, compared to control groups, although these changes did not reach statistical significance. Interestingly, it has been

suggested that during reperfusion, cardiac ATP is partially restored at the expense of ADP and AMP (39,40), which is consistent with the tendency that we observe, for an increased ATP/ADP ratio following application of reperfusion strategies. Nonetheless, there appears to be considerable room for improvement in efficacy of the strategies with respect to ATP repletion. Possible approaches could include perfusate supplementation with stimulators of ATP synthesis, such as adenosine (41), trimetazidine (42) or bendavia (43,44).

We did not observe IR-induced changes in the activities of CII, AK, and CK. These findings are comparable to previous reports in heart homogenates (45) or isolated mitochondria (46). Interestingly, Lee (46) demonstrated that IR modulates the activities of mitochondrial electron transport chain complexes (I-IV) differently. While activities of all complexes (I-IV) were reduced after ischemia, activities of complexes II and IV returned to pre-ischemic levels after 1 hour reperfusion (46). Thus, it could be that at the time of our analysis, after 1 hour reperfusion, ischemia-induced alterations in mitochondrial-related enzymes had already been normalized. Studies aimed to unravel the mechanisms of action at early reperfusion are therefore envisaged.

We monitored three different indicators of mitochondrial mass. While the post-ischemic activities of CS and COX were unchanged between sham and control groups, the mtDNA:nDNA was substantially elevated in control compared to sham hearts. Unchanged mitochondrial enzyme activities are comparable to previous reports (45,46). However, varying effects of cardiac IR on mtDNA have been reported. IR has been associated with mtDNA damage at early reperfusion (47) and upregulation of genes involved in mtDNA replication (45). The IR-induced increase in mtDNA:nDNA that we observe should be interpreted with caution due to the short timeframe of our study (1 hour reperfusion). Unlike the study of Marin-Garcia (45), we did not observe modifications in the activation of the mtDNA transcription. However, the higher release in cyt c that we report after IR does suggest greater mitochondrial damage. Therefore, the increased mtDNA:nDNA in control vs. sham hearts might be a consequence of accumulated mitochondrial damage within the cell,

due to inefficient mitophagy. Indeed, lack of mitophagy has previously been associated with exacerbated IR injury (48,49). Interestingly, in our study, HY significantly increased mRNA levels of Pgc-1α, the master regulator of mitochondrial biogenesis. HY-induced Pgc-1α expression, which has been described in cardiomyocytes after 24 hours exposure to hypoxia (50), is intriguing as a potential contributor to cardioprotection, and may thus prompt further studies.

TBARS and protein carbonylation were investigated as markers of oxidative stress. No difference among experimental groups was observed for TBARS. Indeed, it has been shown that TBARS may provide a better marker of oxidative stress at early reperfusion (51). However, protein carbonylation was significantly increased in control vs. sham hearts, and reperfusion strategies did not limit this increase. Contrary to previous reports (17,22,52), our reperfusion strategies were ineffective against post-ischemic oxidative stress. Thus, the administration of anti-oxidative stress therapies, such as MitoQ (53), in conjunction with our reperfusion strategies may further improve cardioprotective effects through complementary mechanisms.

Correlation analyses between parameters of functional recovery and mitochondrial- and metabolic-related parameters support the notion of differing mechanisms of action for MH, MPC and HY and highlight new, interesting findings. For correlations between 60 min reperfusion parameters, O_2C positively correlated with CO and ATP content for all three strategies, as would be expected with functional mitochondria. Although correlation findings suggest that mitochondrial function is preserved in conjunction with cardiac output for all strategies, diverging correlation patterns for other parameters were revealed. For example, with MH, ATP and PCr correlate positively with recovery of function (LV work and CO), while with MPC, mitochondrial CI and II activities correlate positively with functional parameters (HR, dP/dt_{max}, LV work and CO) and with HY, mitochondrial CII activity correlates negatively with dP/dt_{max}. Further studies are required to investigate the involvement of mitochondrial CII, and its utility as a potential therapeutic target in the setting of both MPC and HY

strategies. For correlation analyses between parameters measured at early reperfusion (10 min) and recovery parameters (at 60 min reperfusion), early mitochondrial damage (determined by cyt c release) does not appear to be associated with recovery parameters in any experimental group. Interestingly, with MPC, early reperfusion O_2:T correlates with measures of contractile recovery; therefore early reperfusion O_2:T may be predictive of recovery of graft function. Also in MPC-treated hearts, early reperfusion TnT release (marker of cell death) positively correlates with several functional parameters, and was significantly increased at 10 min reperfusion compared with controls. Thus, it seems that the MPC-induced myocardial damage at early reperfusion described by Farine (8) may be necessary for subsequent cardioprotection.

We would like to highlight several limitations that apply to our work. Firstly, in order to limit variability and to precisely control our experimental conditions, we used an isolated heart model, which is not fully representative of conditions to which human cardiac grafts are exposed in clinical DCD. Indeed, hearts in our model are not subjected to the catecholamine surge and pulmonary vasoconstriction, previously described in larger animal models of DCD after withdrawal of life-sustaining therapy (54,55) that are particularly damaging for the right ventricle. Furthermore, we must also point-out that our post-ischemic findings can be considered only as short-term recovery as we monitored heart function for a total of one hour after reperfusion onset. This is of particular clinical relevance as graft dysfunction may not develop until several hours post-transplant. In addition, we have included only males in our study in order to avoid gender-related variability in the response to IR injury due to hormonal differences (56). Nonetheless, gender differences are potentially of great relevance in cardioprotection and merit further investigation. Importantly, we consider these findings as a first step towards better understanding of IR injury and its underlying mechanisms. Additional study and confirmation of these findings will be required prior to clinical translation.

In summary, we provide evidence to support the concept that three cardioprotective reperfusion strategies (MH, MPC and HY) act, at least in part, by modifying mitochondrial

function, energy production and/or transfer through differing mechanisms. Although these strategies are effective, room for improvement remains. Indeed, a better understanding of the exact mechanisms of action for each strategy would help to identify new and complementary therapeutic targets, which could lead to the development of more robust reperfusion strategies and, ultimately, aid in the optimization of post-transplant function of DCD hearts.

Acknowledgments

This study was supported by a Project Grant from the Swiss National Science Foundation (310030_149730/1), a Research Grant from the Swiss Heart Foundation and an European Society of Cardiology Research Grant. This article is based upon work from COST Action EU-CARDIOPROTECTION CA16225 supported by COST (European Cooperation in Science and Technology). The authors would like to thank Mr Adrian Segiser for technical support.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.

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Figure legends

Figure 1. Experimental design. Hearts were randomly allocated to one of 5 parallel-arm, experimental groups: 1) control group, hearts were subjected to warm, global ischemia and reperfusion with no cardioprotective strategy; 2-4) treated ischemic groups, immediately following warm, global ischemia one controlled reperfusion strategy (MH, MPC or HY) was applied; and 5) sham group (non-ischemic control), hearts were perfused in aerobic conditions for the entire perfusion period. All hearts initially underwent a 20 min, aerobic baseline period, loaded mode (afterload pressure 80 mmHg) with KHB (see composition in supplemental material) supplemented with 1.2 mM palmitate and 3% albumin. Control, MH, MPC and HY groups were then subjected to 27 min, normothermic global ischemia followed by 60 min reperfusion, while hearts in the sham group were maintained in baseline perfusion conditions for the equivalent time (27 min) to generate non-ischemic control hearts. In all groups, hearts were reperfused for 60 min using KHB without palmitate and albumin supplementation. Reperfusion was initiated in unloaded mode for the first 10 min and then switched to loaded mode for the remaining 50 min (afterload 60 mmHg). Cardioprotective strategies were applied at reperfusion onset as follows: MH, perfusate at 30°C for the first 10 min of reperfusion; MPC, 2 cycles of 30 sec reperfusion and 30 sec ischemia; and HY, perfusate bubbled with 95% N_2/ 5% CO_2 for the first 2 min of reperfusion.

AL, afterload (aortic) pressure; HY, hypoxia; MH, mild hypothermia; MPC, mechanical post-conditioning; R-I, reperfusion-ischemia.

Figure 2. Percent post-ischemic functional and metabolic recovery. Values are presented as 60 min reperfusion values expressed as a percentage of mean baseline values. (A) LV work, left-ventricular work (HR*DP); (B) DP, developed pressure; (C) HR, heart rate; (D) dP/dt_{min}, minimum first derivative of LV pressure; (E) dP/dt_{max}, maximum first derivative of LV pressure; (F) CO, cardiac output; (G) Triple product (LV work*dP/dt_{max}); (H) O_2C, oxygen consumption.

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. sham; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control.

HY, hypoxia; MH, mild hypothermia; MPC, mechanical post-conditioning.

Figure 3. Oxygen metabolism before and after global, warm ischemia. (A) O_2C, oxygen consumption at 20 min baseline and different timepoints during reperfusion (20, 40 and 60 min); (B) O_2:T, oxygen consumption-triple product ratio at 20 min baseline and different timepoints during reperfusion (20, 40 and 60 min reperfusion).

$^{\#}p < 0.05$ vs. sham; *p <0.05 vs. control; **p <0.01 vs. control

HY, hypoxia; MH, mild hypothermia; MPC, mechanical post-conditioning.

Figure 4. Metabolic and cell death markers during reperfusion. (A) TnT release at 10, 20 and 60 min reperfusion; (B) LDH release at 10 and 60 min reperfusion; (C) Cyt c release at 10 and 40 min reperfusion; (D) Lactate accumulation at 10 and 60 min reperfusion.

$^{\#}p < 0.05$ vs. sham; *p <0.05 vs. control.

Cyt c, cytochrome c; HY, hypoxia; LDH, lactate dehydrogenase; MH, mild hypothermia; MPC, mechanical post-conditioning; N.D, non-detectable; TnT, troponin-T.

Figure 5. Cardiac content of high energy metabolites after 60 min reperfusion. (A) ATP, adenosine triphosphate; (B) PCr, phosphocreatine; (C) ADP, adenosine diphosphate; (D) ATP/ADP ratio; all measured in ventricular homogenates.

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. sham; *p <0.05, ***p <0.001 vs. control.

HY, hypoxia; MH, mild hypothermia; MPC, mechanical post-conditioning.

Figure 6. Activity of cardiac enzymes related to energy production after 60 min reperfusion. (A) CI activity, mitochondrial complex I, (B) CII activity, mitochondrial complex II, (C) AK activity, adenylate kinase, and (D) CK activity, creatine kinase; all measured in ventricular homogenates.

$^{\#}p < 0.05$ vs. sham; * p <0.05 vs. control; $^{\&}p < 0.05$ vs. MPC; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs. HY.

HY, hypoxia; IU, international unit; MH, mild hypothermia; MPC, mechanical post-conditioning.

Figure 7. Markers of mitochondrial mass after 60 min reperfusion. (A) CS activity, citrate synthase, (B) COX activity, cytochrome c oxidase, and (C) mtDNA:nDNA, mtDNA-nDNA ratio, measure of relative content of mtDNA; all measured in ventricular homogenates.

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. sham.

HY, hypoxia; IU, international unit; MH, mild hypothermia; MPC, mechanical post-conditioning.

Figure 8. mRNA expression of genes involved in the mitochondrial biogenesis pathway after 60 min reperfusion. (A) Tfam, transcription Factor A, mitochondrial, (B) Nrf1; nuclear respiratory factor 1, (C) Nrf2; nuclear respiratory factor 2, (D) Pgc-1α, peroxisome proliferator-activated receptor-gamma coactivator α and (E) Pgc-1β, peroxisome proliferator-activated receptor-gamma coactivator β; all measured in ventricular homogenates.

* $p < 0.05$ vs. control.

HY, hypoxia; MH, mild hypothermia; MPC, mechanical post-conditioning.

Figure 9. Markers of oxidative stress after 60 min reperfusion. (A) TBARS (thiobarbituric acid reactive substances) content and (B) Oxyblot, protein carbonylation levels after normalization; lower panel, representative oxyblot and Ponceau S images.

$^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. sham; all measured in ventricular homogenates.

AU, arbitrary units; HY, hypoxia; MDA, malondialdehyde; MH, mild hypothermia; MPC, mechanical post-conditioning.

Figure 10. Correlation analysis between recovery, mitochondrial, and metabolic parameters, all measured at 60 min reperfusion. Spearman correlations were performed for (A) pooled control and mild hypothermia groups; (B) pooled control and mechanical post-conditioning groups; (C) pooled control and hypoxia groups. Rho values are indicated by the colour scale. *$p < 0.05$, **$p < 0.01$, $^{£}p < 0.001$.

Mitochondrial related-parameters (Y-axis) measured at 60 min reperfusion: AK, adenylate kinase activity; ATP, adenosine triphosphate; CI, mitochondrial complex I activity; CII, mitochondrial complex II activity; CK, creatine kinase activity; COX, cytochrome c oxidase activity; CS, citrate synthase activity; mtDNA:nDNA, mitochondrial-nuclear DNA ratio; O_2C, oxygen consumption; PCr, phosphocreatine.

Recovery parameters (X-axis): 60 min reperfusion values expressed as percentage recovery of baseline (%): HR, heart rate; DP, developed pressure; dP/dt_{min}, minimum first derivative of LV (left ventricle) pressure; dP/dt_{max}, maximum first derivative of LV pressure; LV work (HR*DP); CO, cardiac output; O_2C, oxygen consumption; O_2:T, oxygen consumption-triple product ratio**.**

Figure 11. Correlation analysis of mitochondrial, metabolic, and cell death measures at 10 min reperfusion with recovery parameters measured at 60 min reperfusion. Spearman correlations were performed for A) pooled control and mild hypothermia groups; (B) pooled control and mechanical post-conditioning groups; (C) pooled control and hypoxia groups. Rho values are indicated by the colour scale. *$p < 0.05$, **$p < 0.01$, $^{£}p < 0.001$.

Early reperfusion parameters (Y-axis) measured at 10 min reperfusion in perfusate samples: cyt c, cytochrome release; LDH, lactate dehydrogenase release; O_2C, oxygen consumption; O_2:T, oxygen consumption-triple product ratio; TnT, troponin-T release.

Recovery parameters (X-axis): 60 min reperfusion values expressed as percentage recovery of baseline (%): AK, adenylate kinase activity; ATP, adenosine triphosphate; CI, mitochondrial complex I activity; CII, mitochondrial complex II activity; CK, creatine kinase activity; CO, cardiac output; COX, cytochrome c oxidase activity; CS, citrate synthase activity; DP, developed pressure; dP/dt_{min}, minimum first derivative of LV (left ventricle) pressure; dP/dt_{max}, maximum first derivative of LV pressure; HR, heart rate; LV work (HR*DP); mtDNA:nDNA, mitochondrial-nuclear DNA ratio; O_2C, oxygen consumption; O_2:T, oxygen consumption-triple product ratio, PCr, phosphocreatine.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure 1

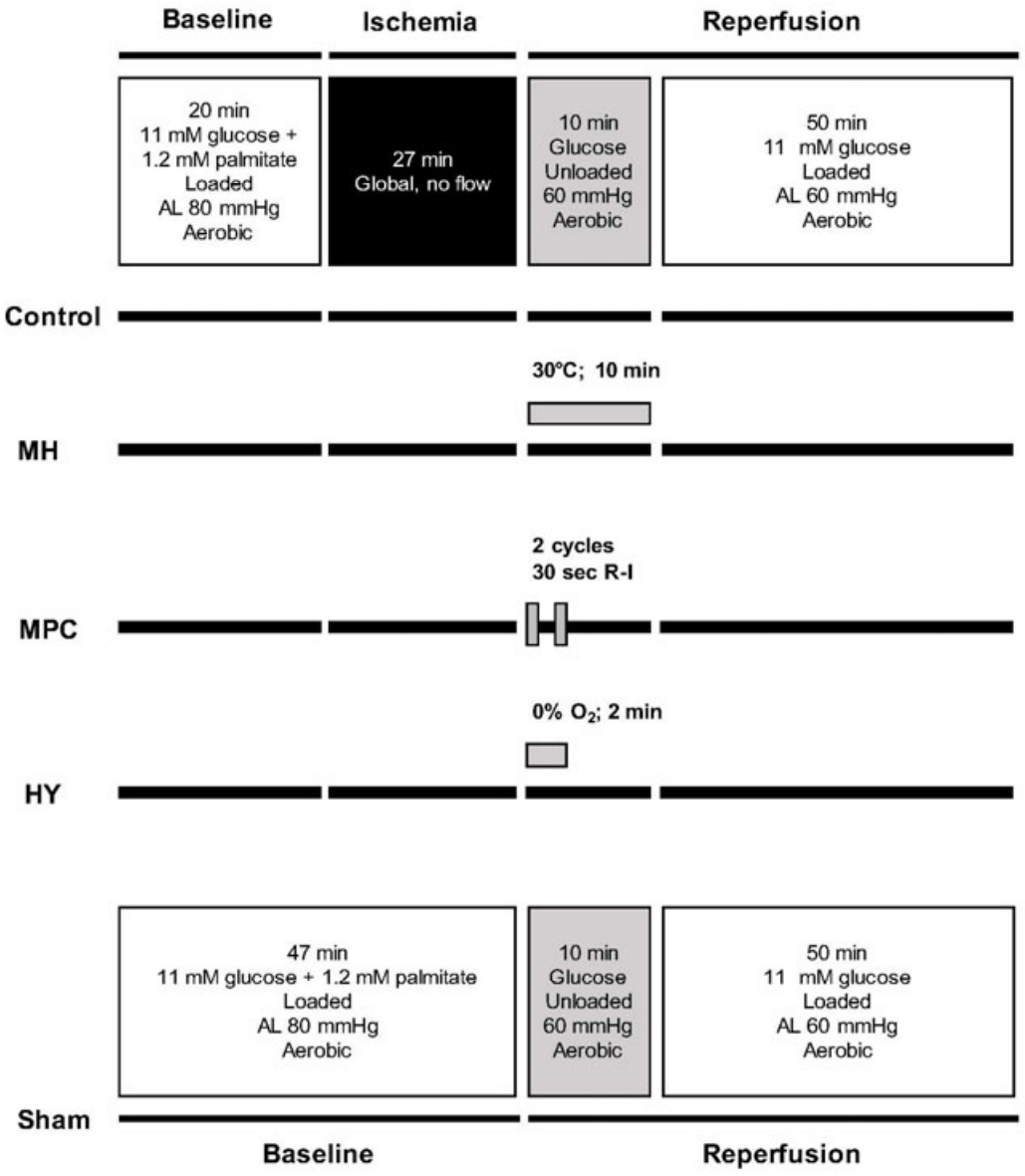


Figure 2

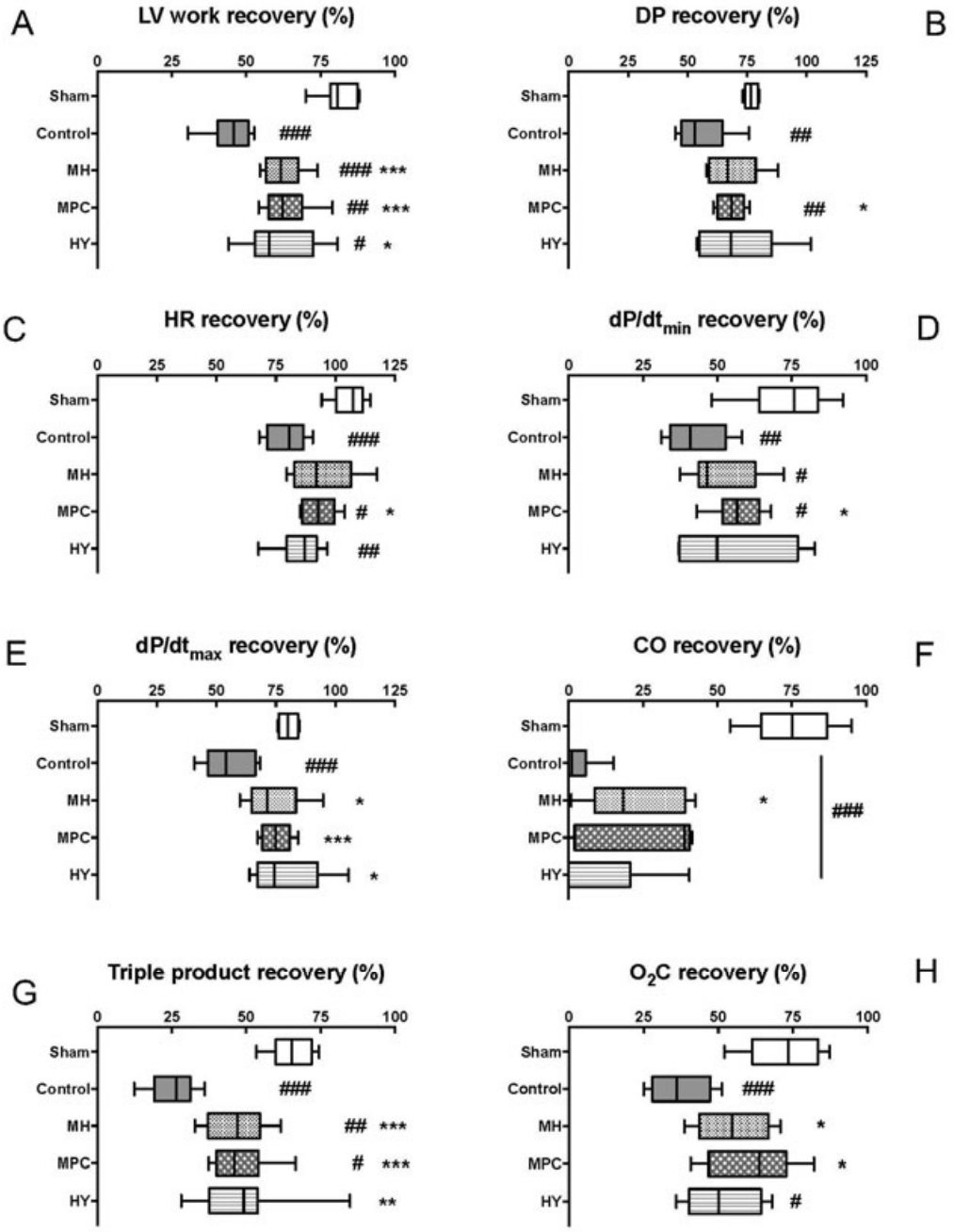


Figure 3

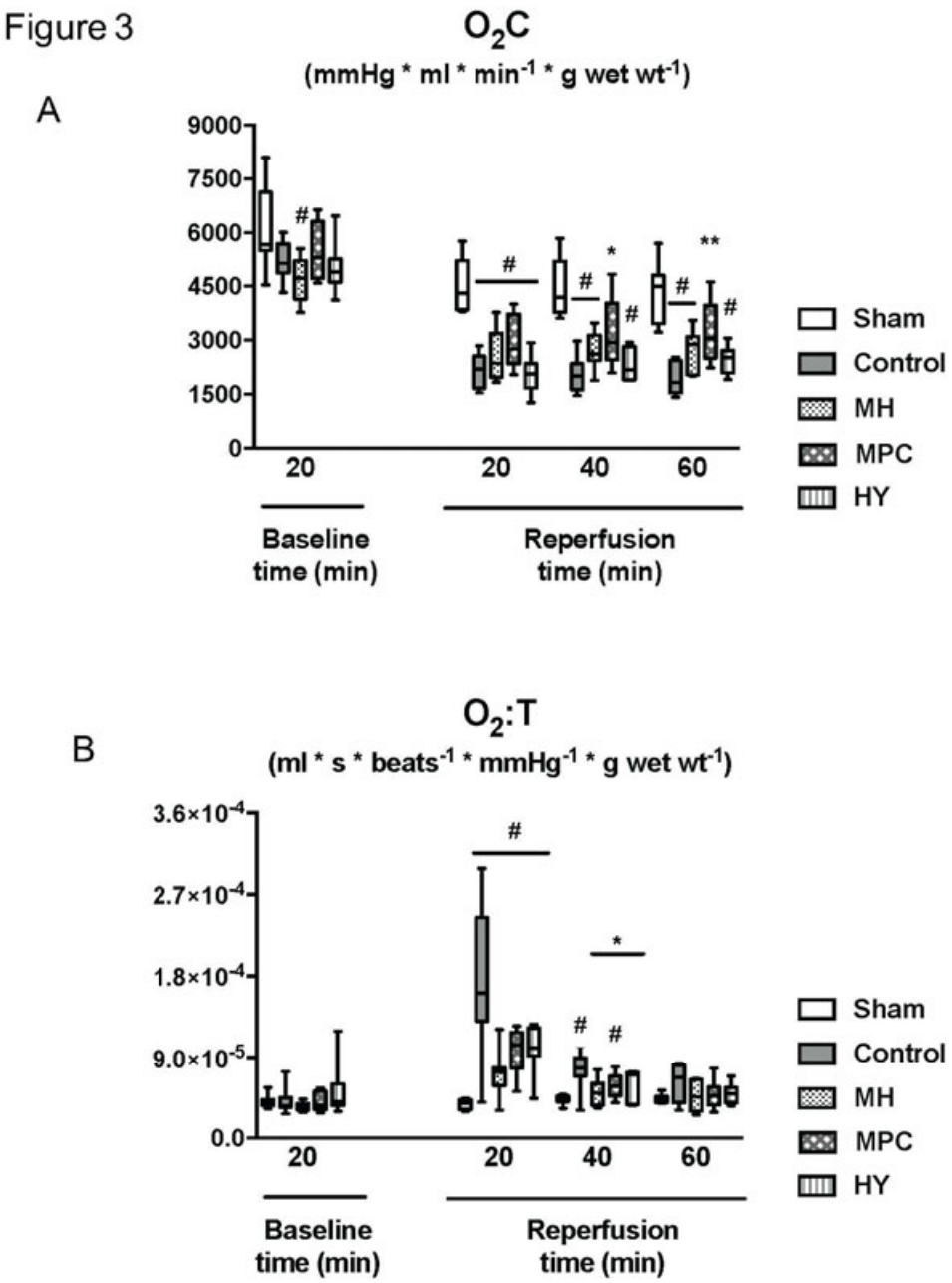
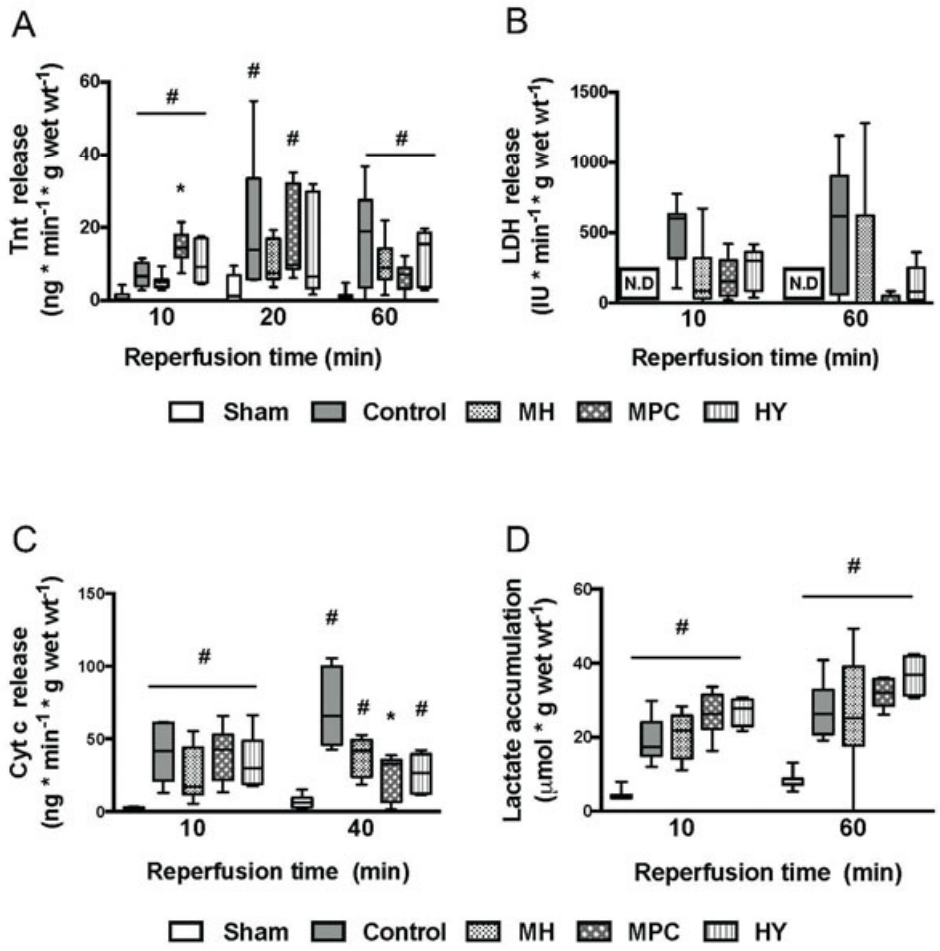


Figure 4



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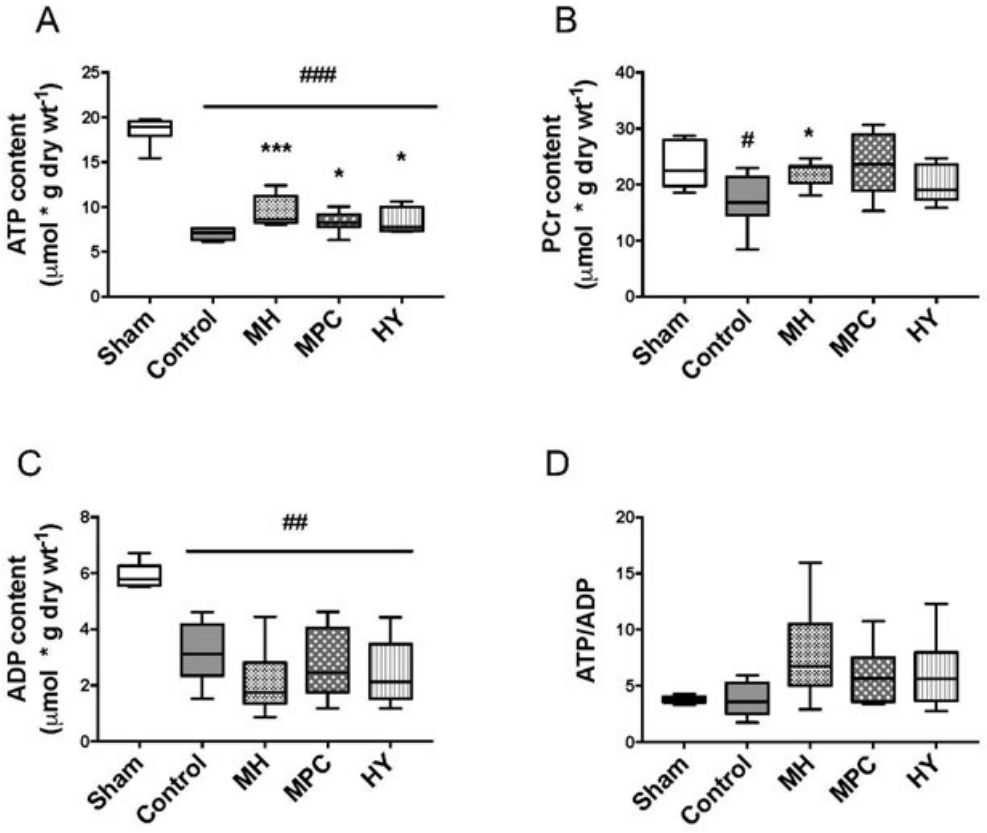

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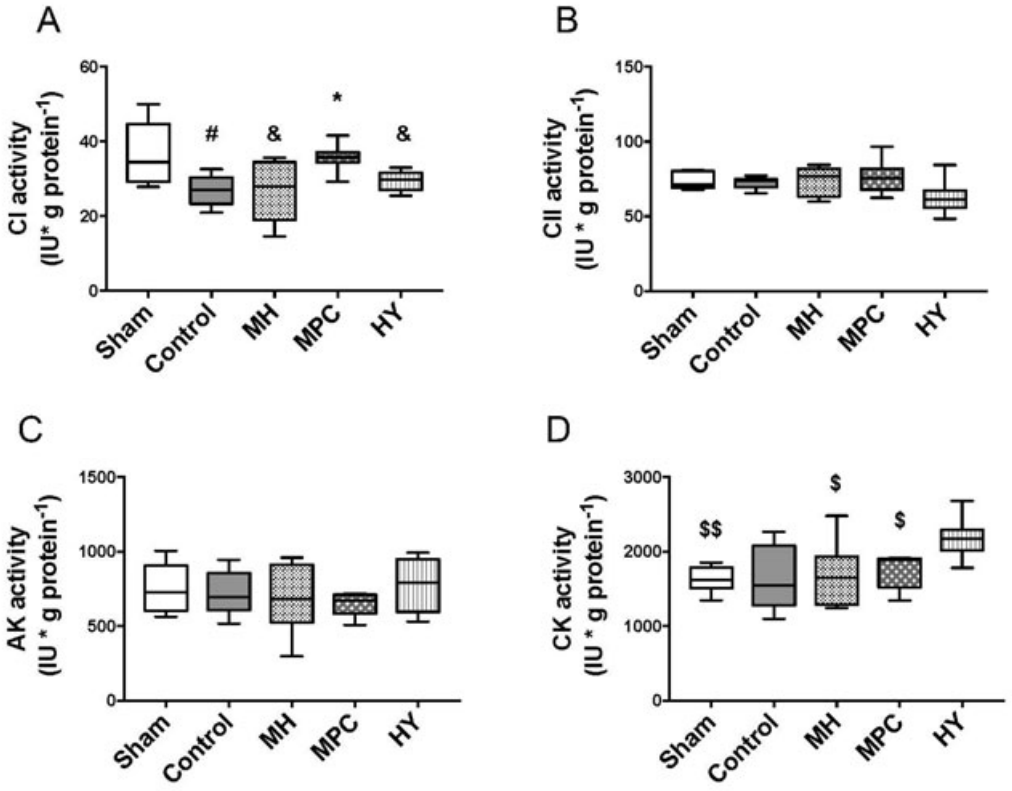

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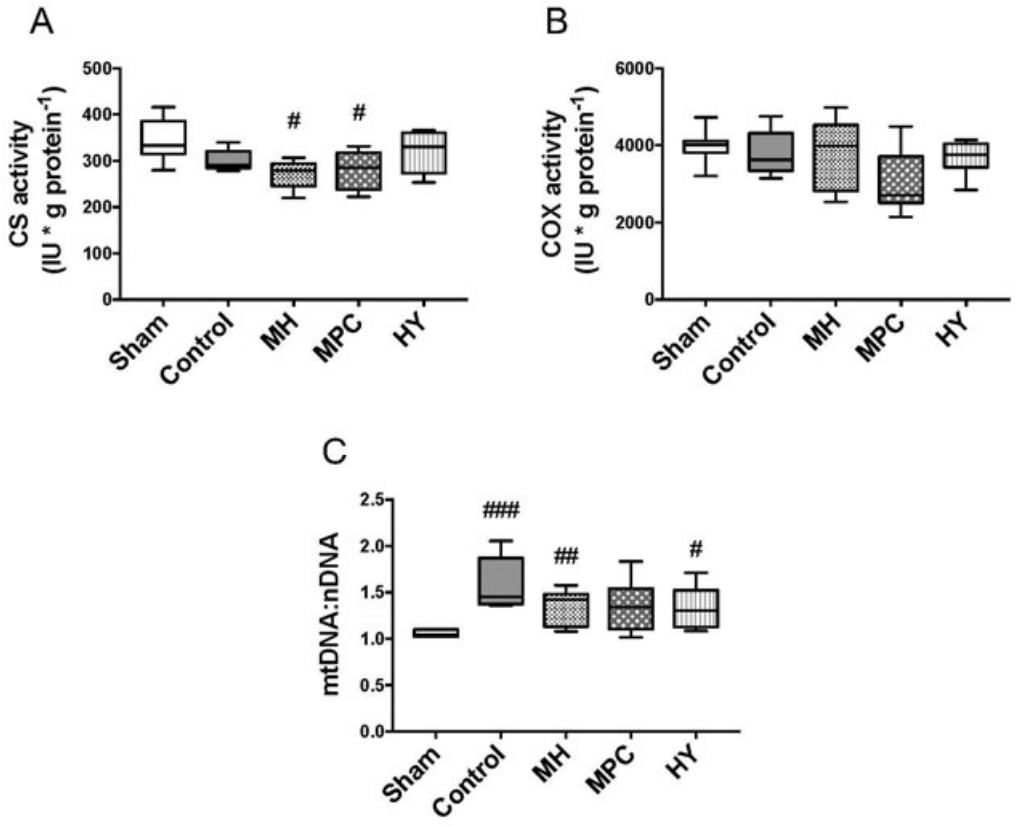

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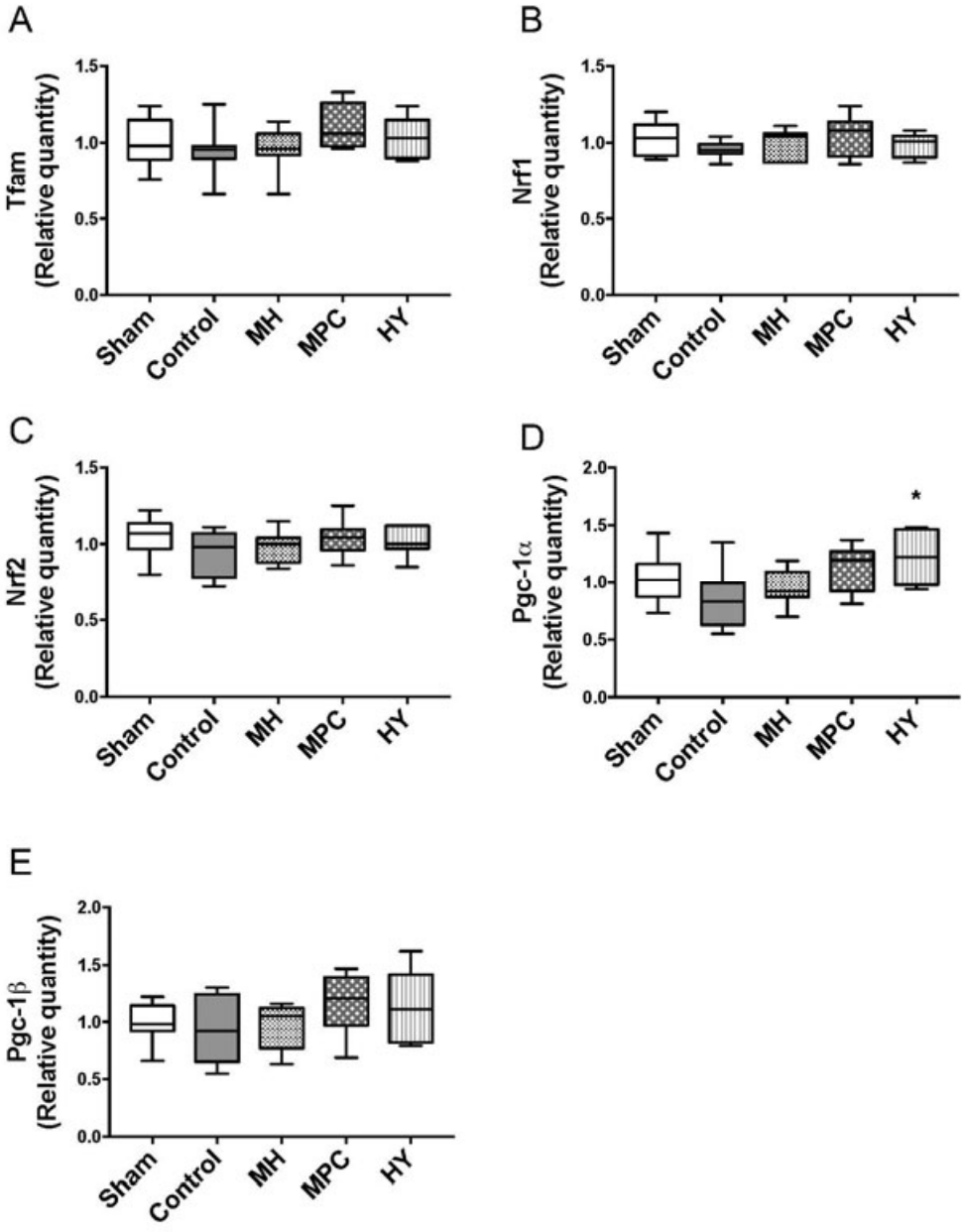

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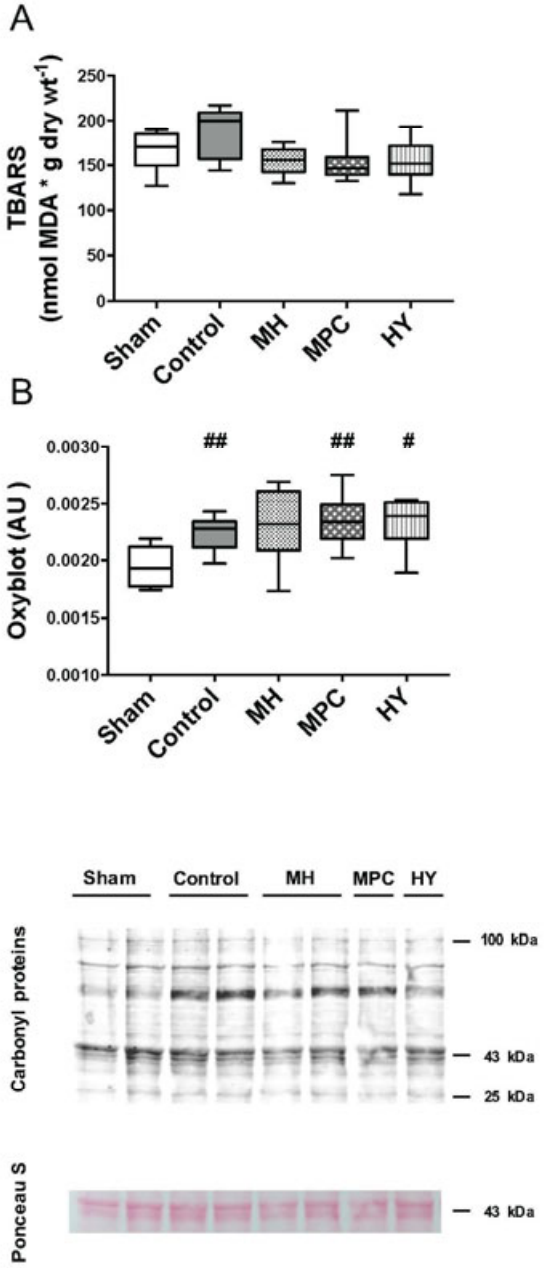

Figure 10

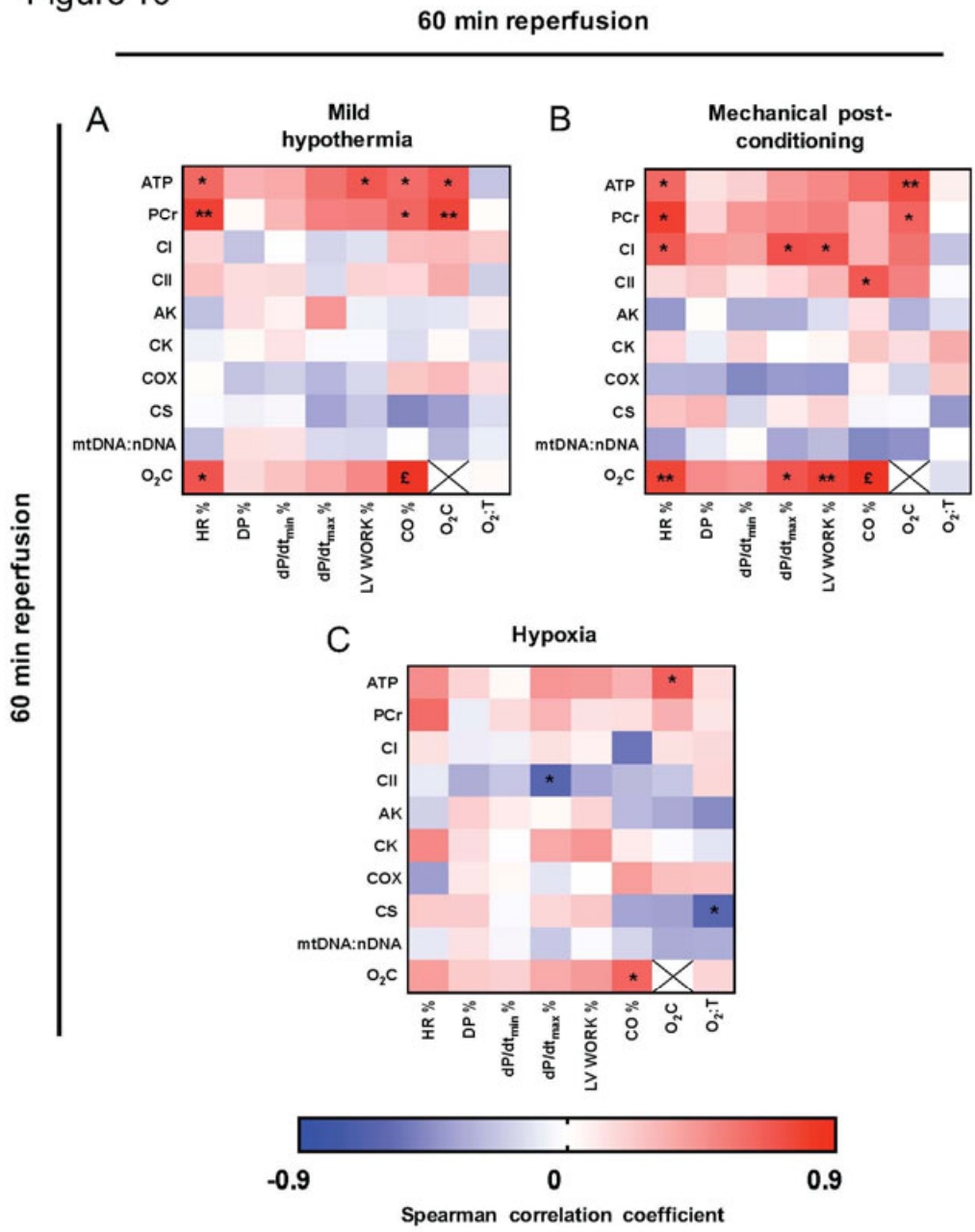

Figure 11