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ORIGINAL RESEARCH

Hypothermic Oxygenated Perfusion Improves Vascular and Contractile Function by Preserving Endothelial Nitric Oxide Production in Cardiac Grafts Obtained With Donation After Circulatory Death

Manuel Egle, MD; Natalia Mendez-Carmona, PhD; Adrian Segiser, MSc; Selianne Graf, MSc, PhD; Matthias Siepe, MD; Sarah Longnus, PhD

BACKGROUND: Cardiac donation after circulatory death is a promising option to increase graft availability. Graft preservation with 30 minutes of hypothermic oxygenated perfusion (HOPE) before normothermic machine perfusion may improve cardiac recovery as compared with cold static storage, the current clinical standard. We investigated the role of preserved nitric oxide synthase activity during HOPE on its beneficial effects.

METHODS AND RESULTS: Using a rat model of donation after circulatory death, hearts underwent in situ ischemia (21 minutes), were explanted for a cold storage period (30 minutes), and then reperfused under normothermic conditions (60 minutes) with left ventricular loading. Three cold storage conditions were compared: cold static storage, HOPE, and HOPE with Nω-nitro-L-arginine methyl ester (nitric oxide synthase inhibitor). To evaluate potential confounding effects of high coronary flow during early reperfusion in HOPE hearts, bradykinin was administered to normalize coronary flow to HOPE levels in 2 additional groups (cold static storage and HOPE with Nω-nitro-L-arginine methyl ester). Cardiac recovery was significantly improved in HOPE versus cold static storage hearts, as determined by cardiac output, left ventricular work, contraction and relaxation rates, and coronary flow ($P<0.05$). Furthermore, HOPE attenuated postreperfusion calcium overload. Strikingly, the addition of Nω-nitro-L-arginine methyl ester during HOPE largely abolished its beneficial effects, even when early reperfusion coronary flow was normalized to HOPE levels.

CONCLUSIONS: HOPE provides superior preservation of ventricular and vascular function compared with the current clinical standard. Importantly, HOPE's beneficial effects require preservation of nitric oxide synthase activity during the cold storage. Therefore, the application of HOPE before normothermic machine perfusion is a promising approach to optimize graft recovery in donation after circulatory death cardiac grafts.

Key Words: donation after circulatory death ■ ex vivo/ex situ heart perfusion ■ heart failure ■ heart transplantation ■ hypothermic oxygenated perfusion

Heart transplantation is the therapeutic gold standard for patients with advanced heart failure.[1] For several years, there has been a significant shortage of hearts available for transplantation, leading to an inability to meet the demand.[2,3] One strategy to improve donor organ availability is heart transplantation

Correspondence to: Sarah Longnus, Department for BioMedical Research, Cardiovascular Disease Program, University of Bern, Murtenstrasse 28, CH-3008 Bern, Switzerland. Email: sarah.henninglongnus@insel.ch

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RESEARCH PERSPECTIVE

What Is New?

- A brief period of hypothermic oxygenated perfusion (HOPE) before normothermic, oxygenated reperfusion improves vascular function, in addition to ventricular function, in cardiac grafts obtained with donation after circulatory death.
- The beneficial effects of HOPE are dependent upon preserved endothelial nitric oxide production.
- Preserved endothelial nitric oxide production leads to reduced tissue calcium overload following normothermic, oxygenated reperfusion.

What Question Should Be Addressed Next?

- Additional research is required to fully understand the mechanisms, effects, and longer-term implications of HOPE to optimize its therapeutic potential in cardiac ischemia and reperfusion.
- New pharmacologic approaches based on nitric oxide or nitric oxide donors could be investigated to further exploit the beneficial mechanisms of HOPE in cardiac ischemia and reperfusion.
- An important next step toward translation to clinical practice is the confirmation of HOPE's superiority over the current clinical standard in a large animal model.

Nonstandard Abbreviations and Acronyms

Akt	protein kinase B
CF	coronary flow
CSS	cold static storage
DCD	donation after circulatory death
eNOS	endothelial nitric oxide synthase
GTN	glyceryl trinitrate
HOPE	hypothermic oxygenated perfusion
L-NAME	Nω-nitro-L-arginine methyl ester
NMP	normothermic machine perfusion
NO	nitric oxide
NOS	nitric oxide synthase

with donation after circulatory death (DCD), which has demonstrated excellent clinical results and relevant increases in transplantation activity in recent years.[4–7]

Despite very promising results with DCD heart transplantation, there are concerns about the inevitable period of global warm ischemia that occurs before organ procurement and may lead to poor organ function.[8,9] Thus, we can now turn our attention to the development of strategies to mitigate ischemia/reperfusion injury.[10] Currently, there are 2 main clinical approaches for DCD heart procurement. One approach is termed *normothermic regional perfusion* (NRP), in which hearts are reperfused in the donor, after the exclusion of cerebral circulation, with the help of extracorporeal membrane oxygenation, and restored to a beating state for evaluation of suitability for transplantation. The second approach includes application of cold preservation solution, followed by direct procurement and perfusion on an ex vivo cardiac perfusion system. With both approaches, the return of oxygenated perfusion to postischemic hearts takes place under normothermic or near-normothermic conditions, which may provoke the development of reperfusion injury, highlighting the need for improved, clinically applicable strategies. Of note, clinical data indicate that 12% to 35% of the harvested DCD heart grafts were not transplanted due to doubts about organ quality.[4,11]

In DCD liver and kidney grafts, it has been shown that hypothermic oxygenated perfusion (HOPE) mitigates reperfusion injury and thus improves graft quality.[12,13] Furthermore, in DCD liver transplantation, the superiority of organ preservation with HOPE over cold static storage (CSS) has been demonstrated at the clinical level.[14,15] Several mechanisms for the beneficial effects of HOPE on ischemia/reperfusion injury in liver and kidney have been proposed,[16] including the reduced reactive oxygen species formation[12,13] and potential preservation of endothelial production of nitric oxide (NO) via both increased endothelial nitric oxide synthase (eNOS) gene expression and enhanced activating phosphorylation.[17,18]

Thus, it is not surprising that HOPE is of great interest in the field of DCD heart transplantation. In a porcine heart model, Moeslund et al demonstrated comparable function among DCD hearts when postischemic normothermic machine perfusion (NMP) was replaced by HOPE.[19] One of the key limitations of HOPE in this context is that hearts remain in a nonbeating state, which significantly limits evaluation possibilities of graft quality. To overcome this problem, our laboratory has developed a new strategy for heart procurement with cardioprotective reperfusion. In the direct procurement and perfusion protocol, the cold ischemia period, which occurs between the application of cold cardioplegia infusion and the onset of NMP and is required for parallel preparation of the heart on a back table and for priming of the perfusion system with blood-buffer mixture, has received rather little attention so far. Wyss et al have investigated replacing this 30-minute period of CSS with HOPE, followed by regular NMP in a rat model, and have demonstrated that this approach can be used to minimize subsequent reperfusion injury.[20] The strategy of using the 30-minute window between cardiac

harvest and warm oxygenated reperfusion for HOPE enables its cardioprotective effects to be used without sacrificing the benefits of NMP, which allows assessment of the graft under beating conditions. However, key mechanisms of HOPE in cardiac preservation remain largely unknown.

To our knowledge, the role of preserved NO production by the application of HOPE and its potential beneficial effects in DCD hearts have not been investigated. Molecular findings regarding NO are of particular interest, as it induces coronary vasodilation but also improves cardiac function. Specifically in the context of ischemia–reperfusion injury, the cardioprotective effect of NO, which occurs by means of multiple overlapping mechanisms, has been demonstrated.[21] We therefore aimed to investigate HOPE in hearts obtained from a DCD rat model by replacing the 30-minute CSS that occurs between the application of cardioplegia and the onset of NMP with HOPE. We hypothesized that preservation of endothelial NO production during the cold preservation period is a key mechanism of HOPE-induced improvements in graft function. This study is of particular interest because it not only tests a clinically applicable therapeutic strategy for its efficacy but also sheds light on its mechanism.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Statement

All experimental procedures were performed in accordance with the European Convention for the Protection of Animals and were approved by the Swiss animal welfare authorities and the State Veterinary Office (Veterinärdiest des Kantons Bern). All surgeries were performed under adequate anesthesia, and all possible efforts were made to minimize animal suffering.

Animals

Male Wistar rats (Janvier Labs, Le Genest-Saint-Isle, France) were housed in groups of 2 under controlled conditions with a 12-hour light–dark cycle and unrestricted access to water and food. To represent young adult DCD heart donors, rats with an age of 11 to 12 weeks and an average weight of 400 g were selected.

Simulation of Clinical DCD Heart Donation With In Situ Ischemia

The DCD rat model established by Arnold et al was used.[22] Based on the results of previous studies, the ischemia time was defined as 21 minutes.[20,23]

Cold Preservation Period

Immediately following heart explantation and aortic cannulation on the perfusion system, a coronary flush with 4 °C St. Thomas N°2 solution supplemented with glyceryl trinitrate (GTN; 0.1 mg/mL) and erythropoietin (5 U/mL) was initiated at a constant pressure of 60 mm Hg for 3 minutes in all hearts. Three different cold storage conditions of 30 minutes were compared: (1) CSS, in which hearts were immersed in 4 °C cold, supplemented with St. Thomas N°2 solution; (2) HOPE with supplemented St. Thomas N°2 (flow, 2.5 mL/min; 10–12 °C; pO_2, 600–700 mm Hg); and (3) HOPE with supplemented St. Thomas N°2, as above, and a pharmacological blocker of nitric oxide synthase (NOS), 10^{-4}M Nω-nitro-L-arginine methyl ester-hydrochloride [L-NAME] (Sigma-Aldrich, St. Louis, MO).

Normothermic Reperfusion

After the cold storage period, hearts were perfused for a total of 1 hour under normothermic (37 °C), aerobic conditions (pO_2, 500–600 mm Hg) with an afterload of 60 mm Hg using a modified Krebs–Henseleit buffer as previously described.[20] In 2 additional groups, bradykinin (Sigma-Aldrich) was administered to hearts undergoing either HOPE and L-NAME or CSS at a concentration in the perfusate of 10^{-6} mol/L to normalize coronary flow (CF) to HOPE levels during early reperfusion. Bradykinin administration was repeated after each 20 minutes. All hearts were perfused in an unloaded (Langendorff) mode for the first 10 minutes, followed by 50 minutes with left ventricular loading. Hearts were then snap-frozen in liquid nitrogen and stored at −80 °C (Figure 1).

Statistical Analysis

A power calculation using means and SDs for the treatment and control groups on the basis of a previous, comparable study[20] was conducted for the primary functional outcomes, namely, left ventricular (LV) work, cardiac output, and CF using an online power calculator provided by the University of British Columbia, Canada (https://www.stat.ubc.ca/~rollin/stats/ssize/n2.html). The analysis showed a required sample size of 2 to 8 (LV work=5, cardiac output=2, CF=8) per group for a power of 0.8 with a 2-sided α of 0.05; thus, we aimed to include 6 to 8 hearts per group.

Unless otherwise stated, values are reported as mean±SD for repeated measures, and as median±interquartile range in box plots. Statistical analyses were performed with Prism software (GraphPad Software, Inc., La Jolla, CA). Data with repeated measurements were analyzed by 1-way ANOVA with repeated measures followed by Fisher's least significant difference test for comparisons between specific groups. To obtain an overview of differences

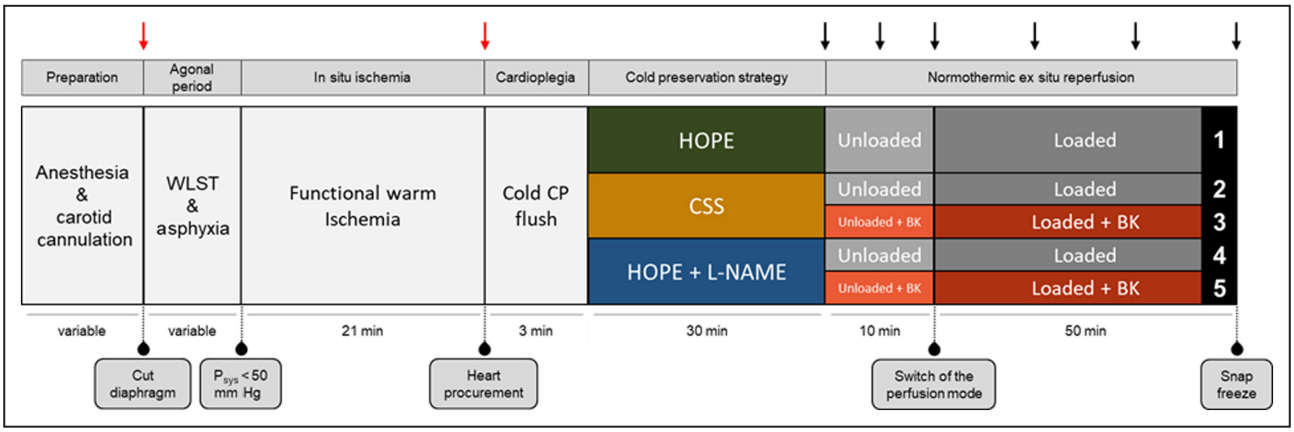

Figure 1. Study design.
This study was performed in a parallel design with 5 experimental arms, as indicated on the right side of the figure. Hearts were subjected to 1 of 3 different cold preservation strategies; CSS, HOPE, and HOPE+L-NAME, while in 2 additional groups (CSS and HOPE+L-NAME), the perfusate was supplemented with bradykinin during reperfusion. Red arrows: blood sample time points, black arrows: perfusate sample time points. BK indicates bradykinin; CP, cardioplegia; CSS, cold static storage; HOPE, hypothermic oxygenated perfusion; L-NAME, Nω-nitro-L-arginine methyl ester; P_{sys}, systolic arterial pressure; and WLST, withdrawal of life-sustaining therapy.

between groups in data with nonrepeated measures, the nonparametric Kruskal–Wallis test was performed. If the result was statistically significant ($P<0.05$), pairwise comparisons were carried out using the Mann–Whitney *U* test. Correlations were analyzed using Spearman's rank correlation test. *P* values were adjusted for multiple comparisons (modified, sequential, rejective Bonferroni procedure).[24] Corrected *P* values are considered statistically significant if <0.05.

Additional Methods

The following methods are described in Data S1: isolated heart preparation, assessment of cardiac function, blood and perfusate sampling, markers of cell death, cytochrome C, tissue calcium, Western blots, and oxidative stress.

RESULTS

Baseline Characteristics

A total of 35 rats in 5 experimental groups (6–8 per group) were included in the study. Baseline characteristics are presented in the Table. There were statistically significant differences in body weight for CSS versus CSS and bradykinin groups and for HOPE and L-NAME versus CSS and bradykinin groups ($P<0.05$ for both). All other values were not statistically different among groups.

Recovery of LV Function After Ischemia

LV function during loaded reperfusion was analyzed by repeated measures ANOVA (Figure 2). HOPE-treated hearts showed significantly ($P<0.05$) better functional

Table. Baseline Characteristics Reported as Median and Interquartile Range

Groups	CSS (n=8)	HOPE (n=7)	HOPE and L-NAME (n=7)	HOPE and L-NAME and bradykinin (n=7)	CSS and bradykinin (n=6)
Rat weight, g	383 (369–398)*	406 (405–414)	392 (369–401)*	400 (392–417)	433 (416–438)
Carotid cannulation time, s	850 (648–945)	770 (645–1072)	630 (595–748)	1015 (798–1184)	802 (700–917)
Mean baseline blood pressure, mm Hg	95.3 (89.3–89.6)	97.6 (93.4–114.7)	102.3 (99.5–114.1)	89.4 (88.1–98.3)	100.4 (97.2–108.6)
Interval WLST to fWIT, s	54 (45–89)	48 (47–189)	73 (54–107)	54 (41–76)	40 (34–81)
Interval fWIT to CA, s	138 (88–175)	199 (90–221)	188 (162–194)	81 (70–146)	96 (39–152)
End ischemia lactate, mmol/L	7.9 (5–10.1)	8.7 (6.4–9.8)	9.2 (7.3–10.3)	9.9 (7.1–11.1)	5.3 (5.3–5.8)

P-values were adjusted for multiple comparisons (modified, sequential, rejective Bonferroni procedure). CA indicates circulatory arrest; CSS, cold static storage; fWIT, functional warm ischemia time; HOPE, hypothermic oxygenated perfusion; L-NAME, Nω-Nitro-L-arginine methyl ester; and WLST, withdrawal of life-sustaining therapy.
*$P<0.05$ vs CSS and bradykinin.

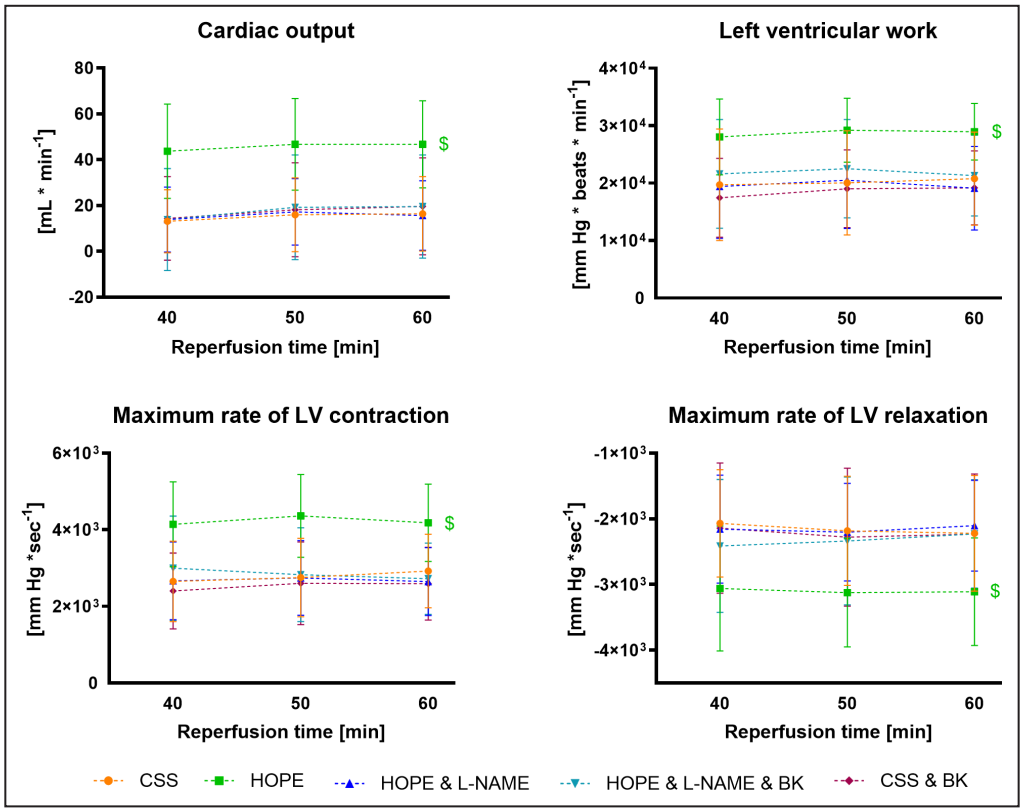

Figure 2. Functional recovery outcomes during normothermic, ex situ reperfusion.
n=6 to 8 per group. $^{\$}P<0.05$ vs all other groups. *P* values were adjusted for multiple comparisons by the modified, sequential, rejective Bonferroni procedure. BK indicates bradykinin; CSS, cold static storage; HOPE, hypothermic oxygenated perfusion; L-NAME, Nω-nitro-L-arginine methyl ester; and LV, left ventricular.

recovery as determined by cardiac output, LV work (heart rate–developed pressure product), maximal contraction and relaxation rates, compared with all other groups. No statistically significant differences were found among any of the other groups.

Recovery of LV Function Correlated With Release of Cell Death Markers

Recovery of LV function, defined as cardiac output or LV work, negatively correlated with the release of the cardiac-specific cell death markers heart-type fatty acid binding protein and troponin I ($P<0.01$ for all; Figure 3). When compared among groups, there was a trend for lower release of heart-type fatty acid binding protein in HOPE-treated hearts compared with all other groups without reaching statistical significance (Figure S1).

Coronary Flow and Coronary Vascular Resistance During HOPE

Coronary vascular resistance during HOPE preservation was significantly lower in HOPE hearts compared with those treated additionally with L-NAME, regardless of subsequent bradykinin treatment ($P<0.05$ for both; Figure 4A). At the end of unloaded perfusion (10 minutes of reperfusion), CF was significantly higher in HOPE hearts compared with CSS and HOPE and L-NAME hearts. The addition of bradykinin to these groups tended to increase CF such that the CFs between these groups and HOPE were no longer significantly different (Figure 4B). During the plateau phase of loaded reperfusion, CF was significantly higher in HOPE compared with all other groups (Figure 4C). Furthermore, CFs were statistically lower in CSS and HOPE and L-NAME groups compared with the corresponding, bradykinin-treated groups. After switching from unloaded to working-heart mode, a statistically significant increase in CF was observed in the HOPE, CSS, and HOPE and L-NAME groups ($P<0.05$; Figure 4D), while the absolute increase was significantly greater in HOPE compared with CSS and HOPE and L-NAME (Figure 4E). Coronary flow measurements at all time points can be found in Figure S2.

Tissue Calcium

Tissue calcium content was generally lower in HOPE hearts compared with the all other groups, reaching

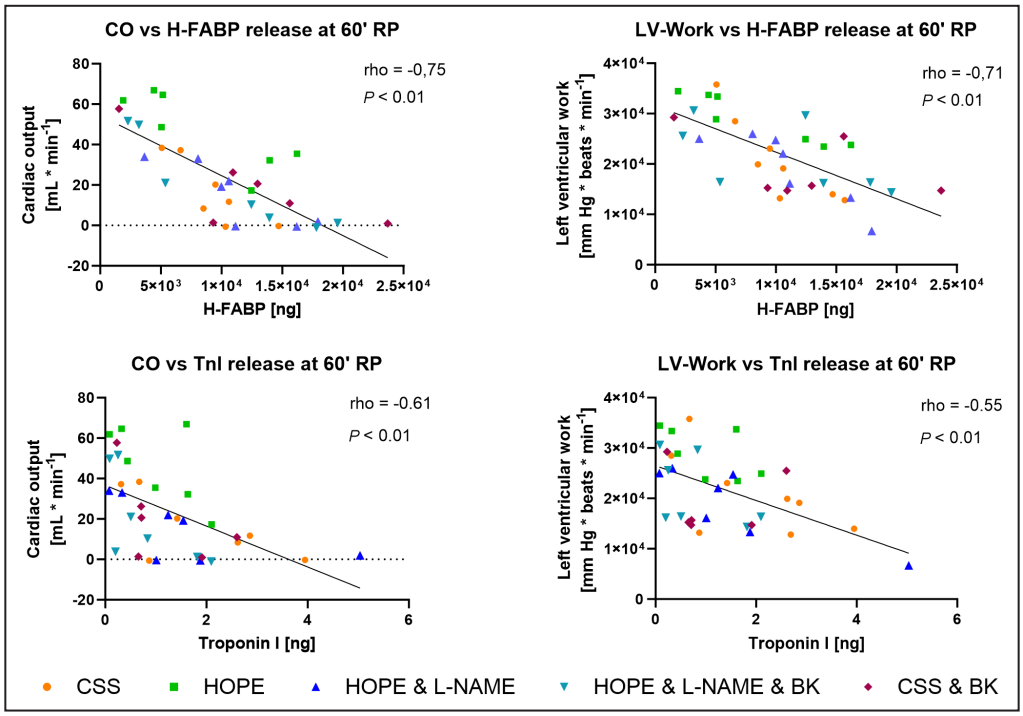

Figure 3. Correlations of ventricular functional outcomes with the release of cell death markers. n=34 to 35. *P* values were adjusted for multiple comparisons by the modified, sequential, rejective Bonferroni procedure. CO indicates cardiac output; H-FABP, heart-type fatty acid binding protein; LV, left ventricular; RP, reperfusion; and TnI, troponin I.

statistical significance when compared with CSS, HOPE and L-NAME and bradykinin or CSS and bradykinin groups ($P<0.01$ for all; Figure 5A). In addition, tissue calcium content significantly correlated with cardiac output (Figure 5B), LV work (Figure 5C), and contractility and relaxation rates (Figure S3). Furthermore, tissue calcium content significantly correlated with the release of heart-type fatty acid binding protein (Figure 5D) and cytochrome C (Figure 5E).

Activation of Key Enzymes

Western blot quantifications showed no statistically significant differences between any groups for eNOS, phospho-eNOS (serine 1177), Akt (protein kinase B), phospho-Akt (serine 473), and the ratios between phosphorylated and nonphosphorylated forms of the enzymes (the latter shown in Figure 6).

Oxidative Stress

Oxidative stress, indicated by protein carbonylation, tended to be increased in the HOPE and L-NAME group, reaching statistical significance compared with HOPE and CSS and bradykinin ($P<0.05$ for both; Figure 7A). A similar pattern was observed for tyrosine nitration, with HOPE and L-NAME levels significantly higher than CSS; HOPE and L-NAME and bradykinin and CSS and bradykinin ($P<0.05$ for all; Figure 7B). In line with these findings, an inverse pattern for total antioxidant capacity was observed with the lowest values for HOPE and L-NAME hearts, reaching statistical significance when compared with CSS or HOPE and L-NAME and bradykinin hearts ($P<0.05$ for both; Figure 7C). In addition, hearts treated with HOPE and L-NAME and bradykinin showed statistically significantly higher total antioxidant capacity compared with HOPE hearts ($P<0.05$). Furthermore, hearts treated with HOPE not only showed significantly higher oxygen consumption compared with all other groups but also showed the highest oxygen efficiency (ratio of cardiac output to oxygen consumption). Details can be found in Figure S4.

Additional results for heart-type fatty acid binding protein and troponin I (Figure S1), CF measurements at all time points (Figure S2), additional correlations with tissue calcium (Figure S3), as well as oxygen consumption and oxygen efficiency (Figure S4) can be found in Data S1.

DISCUSSION

In this preclinical study, we demonstrated that the application of HOPE for only 30 minutes before NMP is a promising reperfusion strategy for providing cardioprotection of DCD hearts versus 30 minutes of CSS,

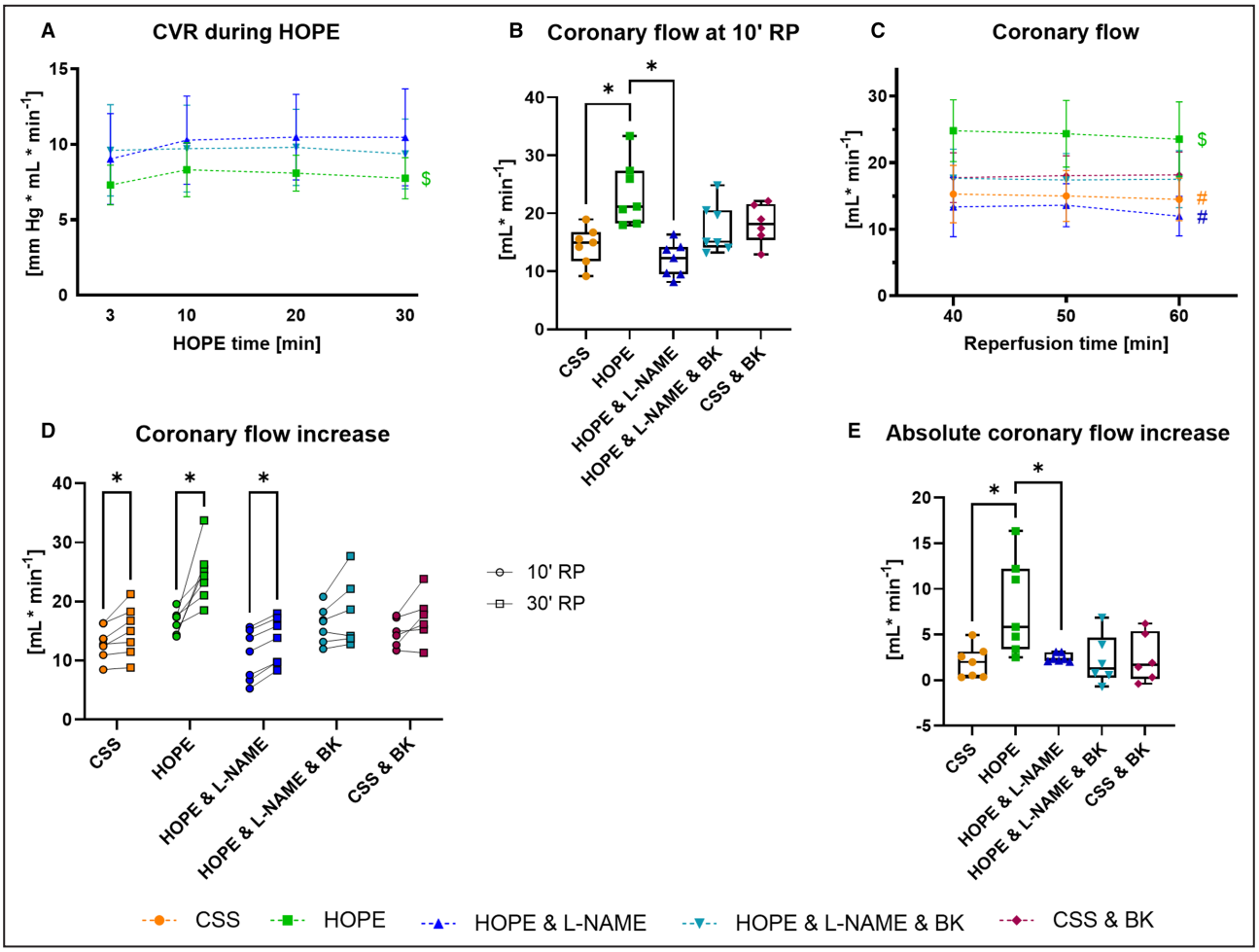

Figure 4. Coronary vascular resistance and coronary flows.
Coronary vascular resistance, measured during the 30 min of cold preservation (**A**). Coronary flow at 10′ unloaded reperfusion (**B**). Coronary flow during plateau phase of loaded reperfusion (**C**). Difference in coronary flow between loaded and unloaded perfusion modes. Unloaded values were measured at 10′ reperfusion and loaded values were measured at 30′ reperfusion (**D**). Absolute increase in coronary flow (**E**). n=6 to 8 per group. $^{*}P<0.05$, $^{\$}P<0.05$ vs all other groups, $^{\#}P<0.05$ vs HOPE & L-NAME & BK and CSS & BK. *P* values were adjusted for multiple comparisons by the modified, sequential, rejective Bonferroni procedure. BK indicates bradykinin; CF, coronary flow; CVR, coronary vascular resistance; CSS, cold static storage; HOPE, hypothermic oxygenated perfusion; L-NAME, Nω-nitro-L-arginine methyl ester; and RP, reperfusion.

the current clinical standard. Furthermore, we present new mechanistic information that reveals a requirement for preserved NOS activity during HOPE for its beneficial effects on cardiac graft recovery. These NO-dependent effects of HOPE include both improved ventricular function, as indicated primarily by greater cardiac output, and improved vascular function, measured by increased active and reactive hyperemic responses. Furthermore, the effects of preserved NO production led to reduced oxidative stress as well as tissue calcium overload, which typically occurs in association with ischemic reperfusion injury. These results are of particular interest, as this therapeutic approach of 30 minutes of HOPE can be incorporated into current clinical practice with NMP, permitting the combination of HOPE's cardioprotective effects with the opportunity to evaluate grafts under beating conditions.

For all parameters of LV function, HOPE hearts showed significantly better recovery compared with all other groups. Strikingly, in hearts that were also exposed to HOPE, but with the addition of the NOS inhibitor, L-NAME, the beneficial effects of HOPE were completely abolished, indicating that NO production plays a key role in the mechanism of HOPE. These results are in line with a previous study from our group demonstrating that the effects of HOPE require the presence of oxygen and are not simply a result of the cold perfusion, as oxygen delivery is required for NO production.[20] Furthermore, we report a relationship between the release of cardiac cell death markers and ventricular recovery, suggesting that the observed differences in functional recovery are not

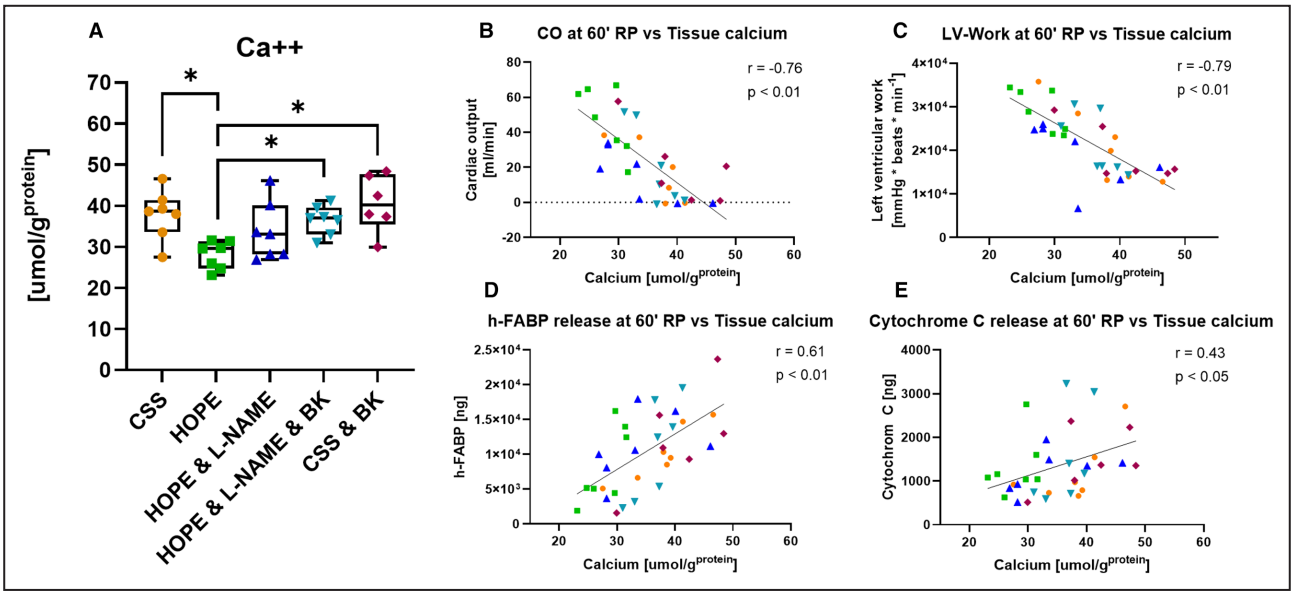

Figure 5. Tissue calcium content

Comparison of tissue calcium content between experimental groups after 60 min of reperfusion, n=6 to 7 per group (A). Correlations between tissue calcium and ventricular function recovery (**B** and **C**), as well as with H-FABP, an indicator of cell death (**D**), and cytochrome C, an indicator of mitochondrial damage (**E**). n=33 to 34 for correlations. **P*<0.05, *P* values were adjusted for multiple comparisons by the modified, sequential, rejective Bonferroni procedure. BK indicates bradykinin; CO, cardiac output; CSS, cold static storage; H-FABP, heart-type fatty acid binding protein; HOPE, hypothermic oxygenated perfusion; L-NAME, Nω-nitro-L-arginine methyl ester; LV, left ventricular; and RP, reperfusion.

simply a transient effect, but are related to protection against reperfusion injury leading to cell death.

HOPE showed NO-dependent improvement in vascular function during cold preservation, early normothermic reperfusion, and LV loading. The measurement of coronary resistance during HOPE allows a first statement regarding endothelial function even before normothermic reperfusion. We have shown that the resistance was higher in hearts in which endothelial NO production was blocked by L-NAME, indicating that NO production during HOPE leads to vasodilation. These results are in agreement with Wyss et al, who report that compared with HOPE conditions, coronary vascular resistance increased during HOPE when oxygen was replaced with nitrogen. Taken together, we can therefore conclude that the lack of increase in coronary vascular resistance provided by HOPE, which is dependent both on the presence of oxygen and activity of NOS, likely depends on the production of NO. During early normothermic reperfusion, HOPE hearts showed significantly increased CF compared with CSS, which has been described as an indicator of improved cardiac function.[25] Furthermore, it was shown that in HOPE hearts, LV loading resulted in a significantly greater increase in coronary flow, compared with CSS hearts, indicating improved recovery of endothelial and vascular function. In HOPE hearts that were additionally treated with L-NAME, early CF was significantly lower compared with HOPE hearts. Moreover, even though CF increased in HOPE and L-NAME hearts with LV loading, the increase was significantly lower compared with HOPE hearts. Thus, our findings during normothermic reperfusion support the concept that HOPE improves reactive and active hyperemic responses in an NO-dependent manner.

Even though early CF is increased by HOPE, this is not sufficient for improved left ventricular and vascular recovery. During early normothermic reperfusion, CF in HOPE and L-NAME was lower versus HOPE hearts. To ensure that the differences in ventricular recovery were dependent on NOS activity inhibition during HOPE, and not persistent L-NAME inhibition of NOS during reperfusion, additional experimental groups with bradykinin treatment during normothermic reperfusion were included. We demonstrated that hearts treated with HOPE and L-NAME had significantly worse recovery of ventricular function despite normalization of early reperfusion CF to HOPE levels, ultimately suggesting that production of NO and its downstream effects during HOPE are necessary for its beneficial effects on vascular function and ventricular recovery.

We also investigated whether HOPE increased activation of the key enzyme eNOS by phosphorylation. In addition, we determined whether HOPE increased phosphorylation/activation of Akt, which in addition to promoting cell survival signaling, triggers the activation of eNOS.[26] Interestingly, our data suggest that neither eNOS nor Akt are stimulated by HOPE after

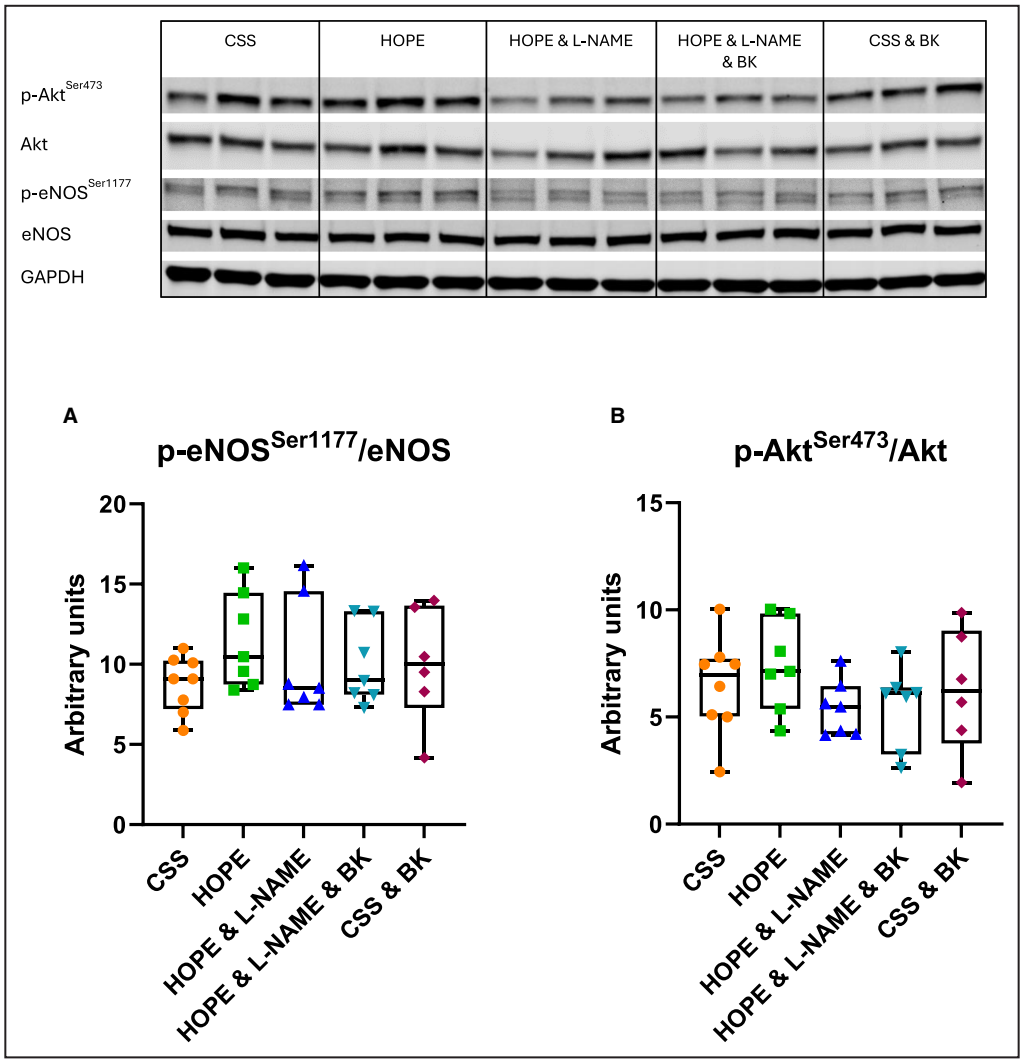

Figure 6. Activation of key pathways
Ratio of eNOS phosphorylated on serine 1177 to total eNOS (A). Ratio of Akt phosphorylated on serine 473 to total Akt (**B**). n=6 to 8 per group. No statistically significant differences were observed among groups (*P* values were adjusted for multiple comparisons by the modified, sequential, rejective Bonferroni procedure). Akt indicates protein kinase B; BK, bradykinin; CSS; cold static storage; eNOS, endothelial nitric oxide synthase; HOPE, hypothermic oxygenated perfusion; L-NAME, Nω-Nitro-L-arginine methyl ester; p-, phosphorylated; and Ser, Serine.

60 minutes of normothermic reperfusion; however, enhanced phosphorylation during and immediately after HOPE cannot be excluded. Thus, it appears that the NOS-dependent beneficial effects on ventricular recovery as well as improved vascular/endothelial function by HOPE are not the result of enhanced activating phosphorylation of NOS or Akt.

NO is known to initiate numerous cellular processes, including activation of the soluble guanylyl cyclase–cGMP–protein kinase G pathway and direct protein nitrosylation, some with cardioprotective effects. One of the key effects of NO is the prevention of ischemia–reperfusion-induced cellular calcium overload by inhibiting the L-type calcium channel.[21] We measured a lower total tissue calcium in HOPE-treated hearts compared with all other groups, reaching statistical significance when compared with CSS or HOPE and L-NAME and bradykinin. In line with these findings, correlations of tissue calcium with several parameters showed statistical significance, including measurements of ventricular function, cell death markers, and the release of cytochrome C. The latter may indicate that the effect of NO associated with HOPE could have an impact on mitochondrial integrity.

In this study, we demonstrate that blocking NO activity during HOPE with L-NAME leads to increased oxidative stress. Our group previously reported that the cardioprotective effects of HOPE in rat hearts require

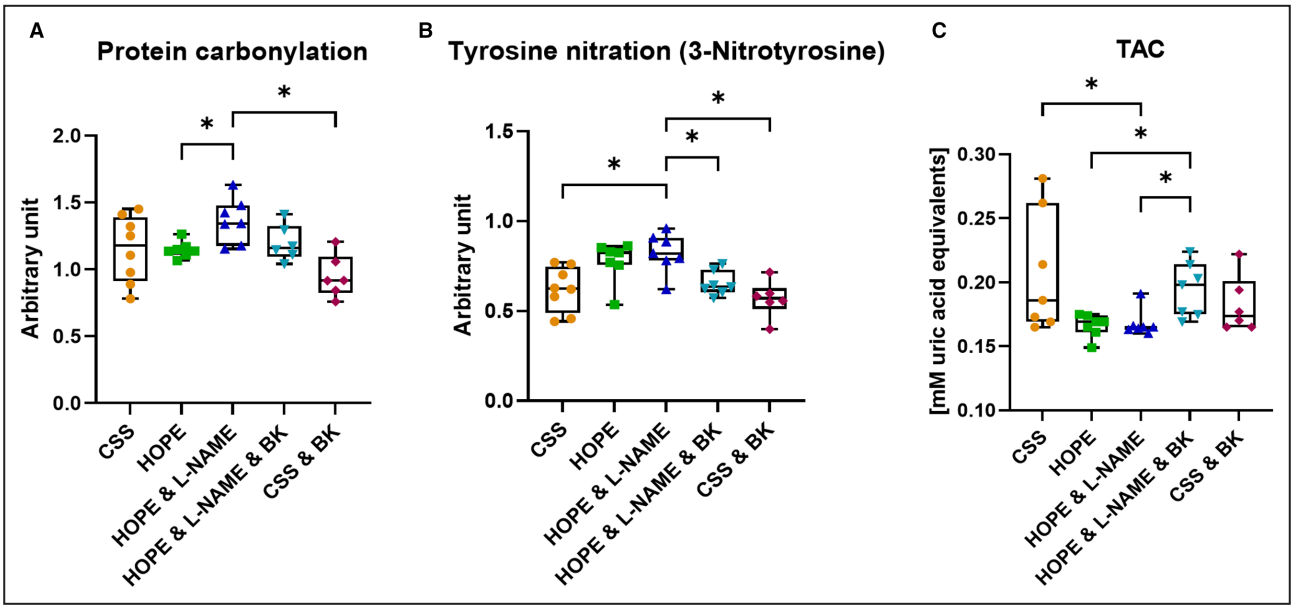

Figure 7. Evaluation of oxidative stress.
The highest values for protein carbonylation as well as tyrosine nitration were measured in HOPE and L-NAME hearts, reaching statistical significance compared with the HOPE and CSS and bradykinin groups for protein carbonylation (**A**) and compared with CSS, HOPE and L-NAME and bradykinin, and CSS and bradykinin for tyrosine nitration (**B**). The lowest values for total antioxidant capacity were measured in HOPE and HOPE and L-NAME hearts; statistically significant differences were observed for HOPE vs HOPE and L-NAME and bradykinin groups and for HOPE and L-NAME vs CSS and HOPE and L-NAME and bradykinin (**C**). n=6 to 8 per group. *P<0.05. P values were adjusted for multiple comparisons by the modified, sequential, rejective Bonferroni procedure. BK indicates bradykinin; CSS, cold static storage; HOPE, hypothermic oxygenated perfusion; L-NAME, Nω-nitro-L-arginine methyl ester; and TAC, total antioxidant capacity.

oxygen; hypothermic, anoxic perfusion does not provide the same benefits.[20] Taken together, these studies support the concept that HOPE-dependent reductions in oxidative stress result, at least in part, from the preservation of NOS activity and production of NO. Consistent with findings of high oxidative stress in the HOPE with L-NAME group, tyrosine nitration was significantly higher, and total antioxidant capacity was significantly lower compared with CSS. Contrary to previous studies in liver, kidney,[13,27] and heart,[20] our data show a picture in which HOPE does not lead to reduced oxidative stress when compared with CSS. It is important to note that effective HOPE treatment is likely achieved by providing the appropriate combination of oxygen delivery and cooling. If either fall above or below the effective range, HOPE may not provide optimal benefits. Furthermore, a minimal time of application is likely required. At present, HOPE conditions that provide the greatest cardioprotection have yet to be defined. It would be of value in future studies to investigate oxygen delivery and cooling thresholds, as well as treatment duration, to optimize HOPE therapy.

The mechanistic insights into the beneficial effects of the preservation of NOS activity, and likely NO itself, in the context of postischemic graft recovery could lead the way to new therapeutic strategies. In the present study, all hearts received the NO-donor GTN in the cardioplegic flush; however, in the CSS hearts, no additional GTN was administered, while in all other groups GTN was continuously administered during the cold preservation period with perfusion. As such, pharmacologic reapplication of NO or NO donors either during graft storage or at the time of reoxygenation should be considered, as this may help to reproduce the beneficial mechanisms of HOPE. However, it is important to note that even in the presence of hypothermic perfusion with the NO donor GTN, oxygen has been previously demonstrated to be required for the protective effects of HOPE.[20] Further studies are needed to investigate the potential beneficial effect of NO therapies in DCD cardiac grafts.

Limitations

Although we used a study protocol that is as clinically relevant as possible, there are some translational limitations of the rat heart model, for example, the absence of blood during NMP. The promising effects of 30-minute HOPE should be investigated in a large-animal model as a next step toward translation into clinical protocols. In the more translational large-animal model, as well as in humans, only dissolved oxygen in the perfusate might not provide sufficient oxygen delivery to achieve beneficial effects of HOPE. Therefore, an

additional oxygen carrier, such as hemoglobin in donor blood, may be necessary.

CONCLUSIONS

With this study, we extend the knowledge of the cardioprotective effects of HOPE in DCD heart transplantation. We confirm that a brief period of HOPE (30 minutes) between heart procurement and NMP results in improved recovery of ventricular function. We report that HOPE also improves recovery of coronary vascular/endothelial function, and that the preservation of NOS activity during HOPE is required for its beneficial effects. In addition, we provide data to support the concept that HOPE-induced ventricular and vascular function improvements do not result simply from increases in early reperfusion CF. Indeed, we provide evidence indicating that reduced calcium overload and oxidative stress appear to be an key mechanisms by which NOS preservation improves cardiac graft quality. Taken together, this study demonstrates a key role for preservation of NOS activity as a mechanism for the beneficial effects of HOPE in DCD heart transplantation. To our knowledge, no clinical data regarding HOPE in hearts with DCD are currently available; our approach is a promising and clinically applicable strategy that merits further investigation toward cardioprotection in the context of DCD heart transplantation with ex vivo graft perfusion.

ARTICLE INFORMATION

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Affiliations

Department of Cardiac Surgery, Inselspital, Bern University Hospital, University of Bern, Switzerland (M.E., N.M., A.S., S.G., M.S., S.L.); Department for BioMedical Research (M.E., N.M., A.S., S.G., S.L.) and Graduate School for Cellular and Biomedical Sciences, University of Bern, Switzerland (M.E., S.G.).

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Dr Egle contributed to all aspects of this manuscript, including planning and designing the study protocol, developing the model, performing experiments, collecting and analyzing the data, and writing the manuscript. Dr Mendez-Carmona, A. Segiser, and Dr Graf participated in the experimental work, data collection, and preparation of the manuscript. Dr Siepe participated in data analysis, preparation of the manuscript, and obtaining funding. Dr Longnus participated in the planning and design of the study, model development, data analysis, preparation of the manuscript, and obtaining funding.

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Disclosures

None.

Supplemental Material

Data S1
Figures S1–S4

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