Author's Accepted Manuscript

Dimethyloxalylglycine Treatment of Brain-Dead Donor Rats Improves both Donor and Graft Left-Ventricular Function after Heart Transplantation

Péter Heged�s MD, Shiliang Li MD, Sevil Korkmaz PhD, Tamás Radovits MD, PhD, Tobias Mayer, Samer Alshaid MD, Paige Brlecic, Matthias Karck MD, Béla Merkely MD, PhD, Gábor Szabó MD, PhD

Cite this article as: Péter Heged�s MD, Shiliang Li MD, Sevil Korkmaz PhD, Tamás Radovits MD, PhD, Tobias Mayer, Samer Alshaid MD, Paige Brlecic, Matthias Karck MD, Béla Merkely MD, PhD, Gábor Szabó MD, PhD, Dimethyloxalylglycine Treatment of Brain-Dead Donor Rats Improves both Donor and Graft Left-Ventricular Function after Heart Transplantation, *J Heart Lung Transplant,* http://dx.doi.org/10.1016/j.healun.2015.06.016

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Dimethyloxalylglycine treatment of brain-dead donor rats improves both donor and graft left-ventricular function after heart transplantation

Péter Hegedűs, MD[1,2], Shiliang Li, MD[1], Sevil Korkmaz, PhD[1], Tamás Radovits, MD, PhD[2], Tobias Mayer[1], Samer Alshaid, MD[1], Paige Brlecic[1], Matthias Karck, MD[1], Béla Merkely, MD, PhD[2], Gábor Szabó, MD, PhD[1]

[1]Department of Cardiac Surgery, University of Heidelberg, Heidelberg, Germany

[2]Heart and Vascular Center, Semmelweis University, Budapest, Hungary

Running Title: DMOG pre-treatment of heart donors

Corresponding Author: Dr. Peter Hegedüs

Laboratory of Cardiac Surgery, Department of Cardiac Surgery

University of Heidelberg, INF 326, 69120 Heidelberg, Germany

Tel.: +49-6221-566246

Fax.: +49-6221-564571

Email:phegedues@gmail.com

1

ABSTRACT

Objective: The hypoxia inducible factor (HIF)-1 pathway signalling has a protective effect against ischemia/reperfusion injury. The prolyl-hydroxylase inhibitor Dimethyloxalylglycine (DMOG) activates the HIF-1 pathway by stabilizing HIF-1α. In a rat model of brain death (BD)-associated donor heart dysfunction we tested the hypothesis that a pre-treatment of brain-dead donors with DMOG results in a better graft heart condition.

Methods: BD was induced in anaesthetized Lewis rats by inflating a subdurally placed balloon catheter. Controls were sham-operated. Then, rats were injected with a single dose of DMOG (30mg/kg i.v.) or an equal volume of physiological saline. After 5h of BD or sham-operation, hearts were perfused with a cold (4°C) preservation solution (Custodiol), explanted, stored at 4°C Custodiol and heterotopically transplanted. Graft function was evaluated 1.5h after transplantation.

Results: Compared to control, BD was associated with decreased left-ventricular systolic and diastolic function. DMOG treatment after BD improved contractility (ESPVR E'$_{max}$: 3.7±0.6 vs 3.1±0.5 mmHg/µl; $p < 0.05$) and left-ventricular stiffness (EDPVR: 0.13±0.03 vs 0.31±0.06 mmHg/µl; $p < 0.05$) 5 hours later compared to brain-dead group. Following heart transplantation, DMOG treatment of brain-dead donors: significantly improved the altered systolic function; decreased inflammatory infiltration; decreased cardiomyocyte necrosis and DNA-strand breakage. Additionally, compared to brain-dead group, DMOG treatment moderated the pro-apoptotic changes in the gene and protein expression.

Conclusions: In a rat model of potential brain-dead heart donors, pre-treatment with DMOG resulted in improved early recovery of graft function after transplantation.

These results support the hypothesis that activation of the HIF-1 pathway has a protective role against BD-associated cardiac dysfunction.

INTRODUCTION

Heart transplantation is the gold standard treatment of end-stage heart failure, presenting excellent results on both short and long term basis [1]. The main limiting factor of organ donation is the availability of suitable organs, meanwhile donor pool is limited to brain-dead donors. Though optimal myocardial preservation is a prerequisite to preserve early allograft function, brain death (BD) itself appears to induce severe changes on the heart function to such an extent, that potential donors must be excluded from transplantation because of emerging hemodynamic instability and primary cardiac dysfunction [2, 3]. Herniation of the brain stem induces a catecholamine storm with an extreme circulatory load, followed by neurohormonal dysfunction with a rapid loss of vasomotoric tone [4]. Abrupt vasodilatation due to circulatory regulational disturbances causes a prominent reduction of coronary blood flow and may result in ischemic injury of the donor heart [5, 6]. The ischemic period activates the hypoxia pathway of the cells via the hypoxia inducible factor (HIF)-1 and its subsequent target genes, such as heme oxygenase (HO)-1, glucose transporter (GLUT)-1, and vascular endothelial growth factor (VEGF). As a result, substrates and enzymes for the anaerobe metabolism are provided, and cell survival is ensured. HIF-1 plays a key role in the regulation of genes responsible for hypoxia adaptation. Though its role in different cellular mechanisms (apoptosis, autophagy) is not yet fully revealed, the activation of HIF-1 improves the ischemic injury of cardiac cells in cell culture [7], and attenuates myocardial ischemia/reperfusion (I/R) injury [8, 9].

Hearts from brain-dead donors exposed to an ischemic event additionally to the transplantational I/R injury, experience a more intense inflammatory response,

3

including the production of cytokines and reactive oxygen and nitrogen species (ROS/RNS), causing endothelial cell dysfunction, disturbance in graft microcirculation, neutrophil extravasation induced inflammatory cascade activation, thus leading to parenchymal tissue damage in the graft [10-12] and ultimately to an increased rate of graft rejection.

Prolyl-4 hydroxylases (PHDs) act as cellular oxygen sensors, and they are the main regulators of (HIF) -1 heterodimer availability. They are responsible for the post-translational HIF-1α hydroxylation under normoxigenic conditions, by targeting the subunit for the von Hippel Lindau tumor suppressor protein E3 ubiquitin ligase complex, thus leading to the degradation of HIF-1α **(Figure 1)** [13]. Dimethyloxallylglycine (DMOG) is the non-selective inhibitor of PHDs. Based on the previously described relations, the aim of the present study was to test the hypothesis that PHD inhibition, by a single dose of DMOG treatment in brain-dead donor rats improves in vivo left ventricular (LV) graft function after a heart transplantation, using our well established model [10, 14].

METHODS

See online supplement for further details.

1.   Animals

Male Lewis rats (250-350g; Charles River, Sulzfeld, Germany) were housed in a room at a constant temperature of 22±2ºC with 12h light/dark cycles and were fed a standard laboratory rat diet with water ad libitum. All animals were cared for in compliance with the 'Principles of Laboratory Animal Care' determined by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised

4

1996) and the German animal protection code. Approval was also granted by the appropriate ethics review board (G-59/14).

2. Experimental groups and timeline

Following a baseline hemodynamic measurement, rats were randomly assigned to three groups: (1) Control group (n=11): sham operated animals without BD induction; (2) Brain-dead group (n=7): after BD induction rats were injected with a vehicle (1ml/kg i.v. physiologic saline); (3) Brain-dead+DMOG group (n=7): after BD induction rats were injected with DMOG (30mg/kg; 1ml/kg i.v.). The experimental protocol is shown in **Figure 2**. Hemodynamic measurements were performed before BD induction, after the induction phase at T=0h and 5 hours later at T=5h. The hearts were then perfused with a cold (4°C) preservation solution (Custodiol), explanted, stored at 4°C Custodiol and then heterotopically transplanted with an ischemic time adjusted to 1h. The LV function was measured after a reperfusion time standardized to 1.5h. The dose of DMOG was chosen on the basis of our pilot studies and data from literature [9, 15, 16].

3. Model of Brain Death

After being anesthetized with sodium pentobarbital (60mg/kg, intraperitoneally), the rats were endotracheally intubated and ventilated by a rodent respirator. For the continuous measurement of arterial pressure, a 2F Millar microtip pressure-volume catheter (SPR-838, Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery. For volume administration purposes, a central venous catheter was inserted into the jugular vein. A small 4F hole was drilled to the bregma, and a balloon catheter was introduced subdurally. BD was induction through gradual

increase in intracranial pressure by inflating the previously inserted balloon with 15µl saline per minute, until a total volume of 750µl was reached. The state of BD was confirmed 30 min after the onset of BD, by the absence of corneal reflexes and a positive apnea test. The blood pressure was maintained through volume administration without inotropic or vasoactive agents. Rats with induced BD and control animals (without BD) were maintained and monitored for 5h.

4. Hemodynamic measurements before, and 5h hours after, sham-operation or brain death induction

In order to assess cardiac function at baseline condition and 5h after the induction of BD, the 2F pressure-volume conductance catheter (Millar Instruments, Houston, TX, USA) was advanced into the left-ventricle. With the use of a special pressure-volume analysis program (PVAN, Millar Instruments, Houston, TX, USA), heart rate, mean arterial pressure, LV end-systolic pressure, LV end-diastolic pressure, LV end-systolic volume, LV end-diastolic volume, stroke volume (SV), maximum rate of rise of LV pressure (dP/dt_{max}), maximum rate of fall of LV pressure (dP/dt_{min}), and the ejection fraction were calculated. Ventricular relaxation was assessed by the time constant of LV pressure decay (Tau), and was calculated using the Glantz method (Tau-g; regression of dP/dt versus pressure). LV pressure-volume relations were measured by transiently occluding the inferior vena cava (reducing preload) under the diaphragm with a cotton-tipped applicator. The slope of the end-systolic pressure-volume relationship (E_{max}; according to the parabolic curvilinear model [17]) was calculated as a load-independent index of LV contractility. The slope of the end-diastolic pressure-volume relationship (EDPVR) was calculated as a reliable index of LV stiffness. At the end of each experiment, 0.1 ml hypertonic saline were injected

into the jugular vein, and from the shift of the pressure-volume relations, the parallel conductance volume was calculated using the software mentioned above. The waws used to correct the cardiac mass volume. After completing the hemodynamic measurements, blood samples were collected from the inferior vena cava. The volume calibration of the conductance system was performed as previously described [18].

5.   **Model of Heterotopic Heart Transplantation**

The transplantations, as previously described [10], were performed in isogenic Lewis to Lewis rat strain, therefore no organ rejection could be expected. The superior and inferior caval veins and the pulmonary veins were tied en masse with a suture and the heart was immediately placed into cold (4ºC) Custodiol solution [19, 20]. The aorta and the pulmonary artery of the donor heart were correspondingly anastomosed end to side to the abdominal aorta and the vena cava of the recipient rat. The ischemic time was adjusted to 1h. After completion of the anastomoses, the heart was reperfused with blood in situ, and the reperfusion time was standardized to 1.5h.

6.   **Measurement of Left-Ventricular Function of the Graft**

One and a half hours after transplantation, a 3F latex balloon catheter (Edwards Lifesciences Corporation, Irvine, CA, USA) was introduced into the left-ventricle via the apex to determine LV systolic pressure, LV diastolic pressure, dP/dt_{max} and dP/dt_{min} by a Millar micromanometer (Millar Instruments, Houston, TX, USA) at different LV volumes. From this data, LV pressure-volume relationships were constructed.

7.   **Histopathological Process**

Heart samples from each experimental group were fixed in buffered paraformaldehyde solution (4%) and embedded in paraffin. Then, 5-µm-thick sections were placed on adhesive slides. Morphometric analysis was performed for inflammation and necrosis on hematoxylin and eosin stained sections. The evaluation was conducted by an investigator blinded to the experimental groups. The evaluation was performed by scoring according to the grade 0-3 under a light optical microscope with nine or ten sections from each sample.

8.   **Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining**

TUNEL staining was performed to detect DNA-strand breaks in the myocardial cells of the left ventricle. TUNEL-positive cells were counted in four fields (characterizing each specimen) under a fluorescence microscope, and an average value was calculated for each experimental group. The evaluation was conducted by an investigator blinded to the experimental groups. Mean values were calculated again for each experimental group, and then were compared.

9.   **Quantitative Real-Time Polymerase Chain-Reaction (PCR)**

Total RNA was isolated from the grafts hearts and then reverse transcription was performed. Quantitative real-time PCR was completed on the LightCycler480 system with the LightCycler480 Probes Master and Universal ProbeLibrary probes (Roche, Mannheim, Germany). Efficiency of the PCR reaction was confirmed with standard curve analysis. Every sample was quantified in duplicate and normalized to

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The expression of HIF-1α and its target genes [HO-1, GLUT-1, VEGF], along with caspase-3, Bcl-2, Nuclear factror (NF)-κβ and Tumor necrosis factor (TNF)-α was determined. Results form every group were illustrated as normalised to the sham-operated control values. For detailed description see online supplementary data and **Online Table 1**.

10. **Western Blotting**

Myocardial protein expression of HIF-1α, HO-1 (1:2000, Abcam, Cambridge, UK), GLUT-1 (1:200, Abcam, Cambridge, UK), cleaved caspase-3 (1:100, NeoMarkers, Fremont CA, USA), and loading control GAPDH (1:500, Santa Cruz Biothechnology, Heidelberg, Germany) was measured by western blot. Results form every group were illustrated as normalised to the sham-operated control values.

11. **Statistical analysis**

All data is tested for normality (Shapiro-Wilk) and expressed as mean ± standard error of the mean (SEM). Intergroup comparisons were performed by using one-way analysis of variance (ANOVA) with Tukey's post hoc test. Differences were considered significant when $p < 0.05$.

RESULTS

LV Cardiac function of donor hearts after brain death

Before the induction of BD (baseline conditions) no significant difference was measured in the hemodynamic parameters of the different groups (data not shown). Mean arterial pressure showed a constant decrease in both brain-dead groups **(Figure 3A)**. Compared either to its baseline or to control group, 5 hours of BD was associated with significantly decreased systolic performance and with an impaired

9

cardiac relaxation. DMOG treatment significantly increased the load-independent contractility parameter (E'_{max}) and improved LV stiffness (slope of EDPVR) of the hearts of brain-dead donors, compared to the untreated brain-dead group **(Table 1)**.

LV function of the heart grafts

Significantly decreased LV systolic pressure, developed LV pressure and dP/dt_{max} were found in both brain-dead groups, when compared to the control rats, indicating decreased systolic function, which was significantly improved by DMOG treatment (**Figures 3B, C, D**). Compared to control, impaired ventricular relaxation was observed in hearts from brain-dead donors. DMOG treatment led to significantly increased dP/dt_{min} and to a lower Tau index (**Figures 3E, F).** LV end-diastolic pressure, as a marker of the standardized balloon-catheter measurements (data not shown) did not show a major difference amongst the experimental groups.

Inflammatory changes of the heart grafts

Semiquantitative histological analysis of the tissue sections revealed a slight infiltration of inflammatory cells, rare necrosis and mild edema in the control group. Similar results were found in the heart grafts after DMOG treatment, while in contrast, extensive inflammation with marked inflammatory cell infiltration, and necrosis were seen in the brain-dead group **(Figures 4A-E)**. A significant up-regulation of transcription factors NF-kB and TNF-α was detected in the brain-dead group when compared to the control group. DMOG treatment significantly decreased the up-regulation of these factors **(Figures 4F, G)**.

DNA-strand breaks and expression of apoptotic factors in the heart-grafts

Increased rate of TUNEL-positive cell nuclei (containing red fluorescent nuclei indicating DNA-fragmentation) was detected in the myocardium of grafts from brain-dead donors when compared to control animals. However, brain-dead donors treated with DMOG were not significantly different from control rats **(Figures 5D)**. In relation to the pro- and anti-apoptotic genes, BD caused a significant up-regulation of caspase-3 and down-regulation of Bcl-2 compared to control, meanwhile DMOG treatment resulted in a significantly lower caspase-3 expression and higher Bcl-2 expression, compared to the brain-dead group **(Figures 5E, F)**. Western-blot analysis of the cleaved caspase-3 levels also supported the results found by quantitative real-time PCR **(Figure 5G)**.

Expressional analysis of the HIF-1 pathway in the heart grafts

Quantitative real-time PCR revealed an up-regulation of the HIF-1α mRNA expression in both groups with BD compared to control group, but it only reached a significant level in the DMOG-treated brain-dead group. In the expression of the HIF target genes HO-1, GLUT-1, and VEGF no difference was observed in the brain-dead group versus the control group, but a considerable up-regulation of these genes was detected after DMOG treatment. Compared to control, BD did not alter the protein levels of HIF-1α, DMOG treatment caused a 3-fold increase in it. Densitometric analysis of the HO-1 bands showed a significant 1.9 fold increase in the brain-dead group compared to control, DMOG treatment further increased its level to 2.5 fold. GLUT-1 protein expression was significantly increased by DMOG treatment, in comparison to control and brain-dead groups **(Figures 6A-H)**.

11

DISCUSSION

In this study the beneficial effect of DMOG pre-treatment against global I/R injury was assessed through a rat model of heterotopic cardiac transplantation using heart grafts from brain-dead donors.

Experimental models demonstrate the widely discussed pathophysiologic consequences of BD [5, 21] and discuss the possible underlying mechanisms, such as catecholamine storm during Cushing-reaction, followed by arterial spasm causing insufficient blood perfusion, or hormonal depletion with abrupt vasodilatation and sudden drop of aortic and coronary blood-pressure [22-25]. In agreement with these findings and in spite of the regularly injected volume, in five hours run MAP significantly decreased along with the LV pressures and load dependent contractility parameters in the brain-dead group **(Table 1)**. Studies even revealed BD induced systemic inflammatory reaction with inflammatory cytokine expression [2, 26]. By these mechanisms BD increases the susceptibility of donor hearts to transplantational I/R injury, and is associated with increased acute rejection rates when the graft organs are derived from brain-dead donor animals [27].

During I/R injury pathologic levels of hypochlorus-acid and peroxynitrite form activated leukocytes cause DNA strand breakage, mitochondrial function disruption, lipid peroxidation induction and depletion of antioxidant reserve, thus activating the apoptotic cascade [28, 29]. Pharmacological preconditioning of cells by the inhibition of oxygen sensors could be a promising strategy against ischemic and inflammatory diseases. The non-selective PHD inhibitor DMOG was previously shown to have advantageous effect against I/R injury in animal models of myocardial infarction [9, 30], and we have recently also reported about the protective effect of DMOG in in vitro vascular function after long term (24h) cold preservation and reperfusion injury

[7]. In their recent publication Keränen et al. described enhanced I/R damage of the graft hearts following HIF activation. This conclusion was drawn from increased cardiac troponin T release and increased inflammatory response in an I/R model following preconditioning of heart donors with PHD inhibitor FG-4497 [31]. Although they did not examine the hemodynamic performance of heart grafts, the contradiction in the results compared to our findings may lay in the different experimental setup and in the different ischemic / reperfusional time intervals.

DMOG pre-treatment of brain-dead donors did not hinder the BD-caused decrease of MAP along with the LV pressures and load dependent contractility parameters in 5h. However, our results show a maintained E'_{max} of ESPVR in the DMOG group, compared to brain-dead group **(Table 1)**. After hypothermic storage and 1.5 hours of reperfusion transplanted hearts from brain-dead donors showed significantly lower contractility and relaxation, which was significantly improved by DMOG pre-treatment **(Figure 3)**. In line with these observations increased DNA strand breakage, up-regulated caspase-3 and TNFα with down-regulated Bcl-2 levels were detected in graft hearts from brain-dead donors following transplantation (caused by the nocuous effect of BD and by the additional I/R injury during transplantation). These changes were moderated due to the advantageous effect of DMOG in the pre-treated group **(Figures 4-5)**.

The mechanisms behind the cardioprotective effects of DMOG against I/R injury are based on the inhibition of the oxygen-sensor PHDs **(Figure 1)**. In the absence of oxygen, PHDs are no longer active and the unmodified HIF-1α accumulates. Dimerising with the constitutive HIF-1β (also known as aryl hydrocarbon receptor nuclear translocator: ARNT), they form HIF-1 heterodimer (a transcription factor of genes participating in tissue response to hypoxia [32]), which limits ischemic or post-

ischemic injury (HO-1, GLUT-1, VEGF, iNOS) [8]. After transplantation, brain-dead donors treated with DMOG showed a significant up-regulation of HIF-1α expression compared to the control group. We also found an up-regulation of HIF-1α mRNA after DMOG treatment compared to control group. However, as there was no difference found when compared to the brain-dead group, this increase could be accounted to the circulatory insufficiency due to BD.

HO-1 overexpression in cardiac tissue leads to a lower apoptosis rate after I/R [33], possible due to its products CO and biliverdin: both of them have been proved to ameliorate I/R injury [34]. GLUT-1 overexpression protected cardiac myocytes from hypoxia induced apoptosis via mitochondrial release of cytochrome c and activation of caspase 9 [35, 36]. VEGF acts as a survival factor along with its long term capillary promoting effect, and in short term decreases the loss of viability, under hypoxic conditions [37, 38]. We confirmed marked up-regulation of these HIF-1 induced protective factors after DMOG treatment **(Figures 6)**.

Expressional changes after the activation of the HIF-pathway during 5 hours incubation contribute to decreased myocardial vulnerability either caused by the damaging effect of BD, or by I/R injury during transplantation. Following these insults, TUNEL is a sensitive method for the assessment of oxidative stress-caused DNA damage. As shown on **Figure 5**, after DMOG pre-conditioning, the lower level of double strand DNA fragmentation is in correlation with the expressional changes in caspase-3 pro- and Bcl-2 anti-apoptotic regulators. From this correlation we conclude a significantly lower apoptotic activity following DMOG pre-treatment compared to that of the brain-dead group.

The silencing of PHDs augments NF-κB activation [39]. Although NF-κB was described to protect myocytes against ischemia-induced apoptosis in a HIF

independent manner by the up-regulation of TNF [40], NF-κB and TNFα also facilitate inflammatory reactions. Under mild hypoxia phosphoinositide-3 kinase/Akt pathway prevents the inhibition of the anti-apoptotic Bcl-xL and induces NF-κB thus leading to cell survival [41]. A significant up-regulation of NF-κB and TNFα mRNA were detected along with increased infiltration of inflammatory cells in the brain-dead group compared to control and DMOG.

All together, in clinical perspective DMOG treatment may have advantegous effect under brain-dead state, protecting against the ischemic events and inflammation-caused oxidative load. On the other hand, DMOG pre-conditioning a couple of hours before transplantation could improve early post-transplantational graft-function.

Conclusions

To the best of our knowledge, our study is the first that records the cardioprotective effect of a pharmacological preconditioning with dimethyloxalylglycine on the donor heart function under BD conditions, and on the LV graft function after transplantation. The treatment of brain-dead heart donors with DMOG resulted in a significantly better left-ventricular function after the transplantation. All in all, our results support the view that preconditioning through the inhibition of oxygen sensors, and through the activation of HIF-1 pathway is, potentially, at least in part, associated with a protective role against I/R injury in the myocardium.

Study limitations

The model of heterotopic heart transplantation also has certain limitations. The left-ventricle beats in an unloaded condition (e.g. ventricles are perfused via the coronary circulation, but they do not eject) which on one hand allows a faster recovery after transplantation, and on the other hand leads to a time-dependent mechanical deterioration and atrophy (though it does not occur in 24h) [42]. The influence of prolonged anesthesia on the donor cardiac function shall also be considered a limitation of the model of BD as it deviates from the clinical setting of most donors.

The brief period of reperfusion (1.5 hrs) limits conclusions regarding the effects of DMOG to early recovery of donor function from BD-associated and I/R-induced injuries. Prudent interpretation of the findings obtained from this animal model is required for the extrapolation to humans.

Conflict of interest

None.

REFERENCES

1. Kransdorf, E.P. and J. Stehlik, *Donor evaluation in heart transplantation: The end of the beginning.* J Heart Lung Transplant, 2014. **33**(11): p. 1105-13.
2. Szabo, G., *Physiologic changes after brain death.* Journal of Heart and Lung Transplantation, 2004. **23**(9): p. S223-S226.
3. Birks, E.J., et al., *Activation of apoptotic and inflammatory pathways in dysfunctional donor hearts.* Transplantation, 2000. **70**(10): p. 1498-1506.
4. van der Hoeven, J.A.B., et al., *Effects of brain death and hemodynamic status on function and immunologic activation of the potential donor liver in the rat.* Annals of Surgery, 2000. **232**(6): p. 804-812.
5. Herijgers, P., et al., *Changes in organ perfusion after brain death in the rat and its relation to circulating catecholamines.* Transplantation, 1996. **62**(3): p. 330-335.
6. Szabo, G., et al., *Brain death impairs coronary endothelial function.* Transplantation, 2002. **73**(11): p. 1846-1848.
7. Barnucz, E., et al., *Prolyl-hydroxylase inhibition preserves endothelial cell function in a rat model of vascular ischemia reperfusion injury.* J Pharmacol Exp Ther, 2013. **345**(1): p. 25-31.
8. Natarajan, R., et al., *Hypoxia inducible factor-1 activation by prolyl 4-hydroxylase-2 gene silencing attenuates myocardial ischemia reperfusion injury.* Circulation Research, 2006. **98**(1): p. 133-140.
9. Ockaili, R., et al., *HIF-1 activation attenuates postischemic myocardial injury: role for heme oxygenase-1 in modulating microvascular chemokine generation.* American Journal of Physiology-Heart and Circulatory Physiology, 2005. **289**(2): p. H542-H548.
10. Szabo, G., et al., *Poly(ADP-ribose) polymerase inhibition reduces reperfusion injury after heart transplantation.* Circulation Research, 2002. **90**(1): p. 100-106.
11. Zweier, J.L. and M.A.H. Talukder, *The role of oxidants and free radicals in reperfusion injury.* Cardiovascular Research, 2006. **70**(2): p. 181-190.
12. Boyle, E.M., et al., *Endothelial cell injury in cardiovascular surgery: Ischemia-reperfusion.* Annals of Thoracic Surgery, 1996. **62**(6): p. 1868-1875.
13. Piret, J.P., et al., *Is HIF-1alpha a pro- or an anti-apoptotic protein?* Biochem Pharmacol, 2002. **64**(5-6): p. 889-92.
14. Li, S.L., et al., *Acute Ethanol Exposure Increases the Susceptibility of the Donor Hearts to Ischemia/Reperfusion Injury after Transplantation in Rats.* Plos One, 2012. **7**(11).
15. Ogle, M.E., et al., *Inhibition of prolyl hydroxylases by dimethyloxaloylglycine after stroke reduces ischemic brain injury and requires hypoxia inducible factor-1alpha.* Neurobiol Dis, 2012. **45**(2): p. 733-42.
16. Yuan, Q., et al., *PHDs inhibitor DMOG promotes the vascularization process in the AV loop by HIF-1a up-regulation and the preliminary discussion on its kinetics in rat.* BMC Biotechnol, 2014. **14**(1): p. 961.
17. Kass, D.A., et al., *Influence of contractile state on curvilinearity of in situ end-systolic pressure-volume relations.* Circulation, 1989. **79**(1): p. 167-78.
18. Pacher, P., et al., *Measurement of cardiac function using pressure-volume conductance catheter technique in mice and rats.* Nature Protocols, 2008. **3**(9): p. 1422-1434.
19. Li, S., et al., *Short- and long-term effects of brain death on post-transplant graft function in a rodent modeldagger.* Interact Cardiovasc Thorac Surg, 2015. **20**(3): p. 379-86.
20. Li, S., et al., *Transplantation of donor hearts after circulatory or brain death in a rat model.* J Surg Res, 2014.
21. Galinanes, M. and D.J. Hearse, *Brain death-induced impairment of cardiac contractile performance can be reversed by explantation and may not preclude the use of hearts for transplantation.* Circ Res, 1992. **71**(5): p. 1213-9.

22. Apostolakis, E., H. Parissis, and D. Dougenis, *Brain death and donor heart dysfunction: implications in cardiac transplantation.* J Card Surg, 2010. **25**(1): p. 98-106.

23. Banki, N.M., et al., *Acute neurocardiogenic injury after subarachnoid hemorrhage.* Circulation, 2005. **112**(21): p. 3314-9.

24. Bittner, H.B., et al., *A valid experimental brain death organ donor model.* J Heart Lung Transplant, 1995. **14**(2): p. 308-17.

25. Kolin, A. and J.W. Norris, *Myocardial damage from acute cerebral lesions.* Stroke, 1984. **15**(6): p. 990-3.

26. van Der Hoeven, J.A., et al., *Effects of brain death and hemodynamic status on function and immunologic activation of the potential donor liver in the rat.* Ann Surg, 2000. **232**(6): p. 804-13.

27. Wilhelm, M.J., et al., *Activation of the heart by donor brain death accelerates acute rejection after transplantation.* Circulation, 2000. **102**(19): p. 2426-2433.

28. Zweier, J.L. and M.A. Talukder, *The role of oxidants and free radicals in reperfusion injury.* Cardiovasc Res, 2006. **70**(2): p. 181-90.

29. Korkmaz, S., et al., *Dose-dependent effects of a selective phosphodiesterase-5-inhibitor on endothelial dysfunction induced by peroxynitrite in rat aorta.* Eur J Pharmacol, 2009. **615**(1-3): p. 155-62.

30. Fraisl, P., J. Aragones, and P. Carmeliet, *Inhibition of oxygen sensors as a therapeutic strategy for ischaemic and inflammatory disease.* Nat Rev Drug Discov, 2009. **8**(2): p. 139-52.

31. Keranen, M.A., et al., *Differential effects of pharmacological HIF preconditioning of donors versus recipients in rat cardiac allografts.* Am J Transplant, 2013. **13**(3): p. 600-10.

32. Wenger, R.H., *Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression.* FASEB J, 2002. **16**(10): p. 1151-62.

33. Katori, M., et al., *Heme oxygenase-1 overexpression protects rat hearts from cold ischemia/reperfusion injury via an antiapoptotic pathway.* Transplantation, 2002. **73**(2): p. 287-92.

34. Nakao, A., et al., *Protection against ischemia/reperfusion injury in cardiac and renal transplantation with carbon monoxide, biliverdin and both.* Am J Transplant, 2005. **5**(2): p. 282-91.

35. Malhotra, R. and F.C. Brosius, 3rd, *Glucose uptake and glycolysis reduce hypoxia-induced apoptosis in cultured neonatal rat cardiac myocytes.* J Biol Chem, 1999. **274**(18): p. 12567-75.

36. Malhotra, R., et al., *Hypoxia-inducible factor-1alpha is a critical mediator of hypoxia induced apoptosis in cardiac H9c2 and kidney epithelial HK-2 cells.* BMC Cardiovasc Disord, 2008. **8**: p. 9.

37. Messadi, E., et al., *Cardioprotective Effect of VEGF and Venom VEGF-like Protein in Acute Myocardial Ischemia in Mice: Effect on Mitochondrial Function.* J Cardiovasc Pharmacol, 2014. **63**(3): p. 274-81.

38. Yang, J., et al., *Neuroprotective effects of VEGF administration after focal cerebral ischemia/reperfusion: dose response and time window.* Neurochem Int, 2012. **60**(6): p. 592-6.

39. Robinson, A., et al., *Mucosal protection by hypoxia-inducible factor prolyl hydroxylase inhibition.* Gastroenterology, 2008. **134**(1): p. 145-55.

40. Misra, A., et al., *Nuclear factor-kappaB protects the adult cardiac myocyte against ischemia-induced apoptosis in a murine model of acute myocardial infarction.* Circulation, 2003. **108**(25): p. 3075-8.

41. Alvarez-Tejado, M., et al., *Hypoxia induces the activation of the phosphatidylinositol 3-kinase/Akt cell survival pathway in PC12 cells: protective role in apoptosis.* J Biol Chem, 2001. **276**(25): p. 22368-74.

42. Galinanes, M. and D.J. Hearse, *Metabolic, functional, and histologic characterization of the heterotopically transplanted rat heart when used as a model for the study of long-term recovery from global ischemia.* J Heart Lung Transplant, 1991. **10**(1 Pt 1): p. 79-91.

Table 1.: Cardiac hemodynamic parameters of donors 5h after sham operation or induction of brain death

Parameters	Control donors	Brain-dead donors	Brain-dead+DMOG donors
Heart rate (beats/min)	384±15	351±19	372±21
MAP (mmHg)	95±5	**51±3***	**48±5***
LVESP (mmHg)	109±6	**57±6***	**64±7***
LVEDP (mmHg)	9±2	**6±1***	**4±1***
Ejection fraction (%)	42±5	**25±5***	**26±4***
dP/dt$_{max}$ (mmHg/s)	7940±765	**3418±560***	**3086±418***
dP/dt$_{min}$ (mmHg/s)	10396±663	**3121±328***	**3138±860***
LVESV (µl)	58±19	**115 ±28***	**105±22***
LVEDV (µl)	83±18	**146±29***	**136±22***
Stroke Volume (µl)	40±4	36±8	35±5
ESPVR E'$_{max}$ (mmHg/µl)	3.5±0.4	3.1±0.5	**3.7±0.6[#]**
Tau-g (ms)	10±0.5	15±6	15±4
Slope of EDPVR (mmHg/µl)	0.112±0.02	**0.301±0.06***	**0.128±0.03[#]**

MAP: mean arterial pressure, LVESP: left-ventricular end-systolic pressure; LVEDP: left-ventricular end-diastolic pressure, dP/dt$_{max}$: maximum rate of rise of LV pressure, dP/dt$_{min}$: maximum rate of fall of LV pressure, LVESV: left-ventricular end-systolic

volume, LVEDV: left-ventricular end-diastolic volume, EDPVR: end-diastolic pressure volume relationships. All values are expressed as mean±SEM. *p<0.05 versus Control, #p<0.05 versus BD.

Figure 1. The HIF-pathway and the role of DMOG

HIF: Hypoxia Inducible Factor; PHDs: members of Prolyl-Hydorxilase family; OH: hydoxil-group and pVHL: Von Hippel-Lindau protein.

Figure 2. Experimental protocol

Hemodynamic functional measurements were performed before BD induction (baseline), after confirmation of BD or after sham-operation with injection of Saline or DMOG at T=0 hours, and at T=5 hours after BD induction. The left-ventricular function of the graft hearts was measured 1.5 hours after the initiation of reperfusion.

Figure 3. Mean arterial pressure during brain-dead state (A) and

Left-ventricular function of the graft hearts after transplantation (B-F)

(A) Mean arterial pressure volumes during the 5 hours of BD (B) Left-ventricular end-systolic pressure - left-ventricular volume (LVESP); (C) Left-ventricular (LV) developed pressure; (D) Change of mean arterial pressure during BD; (E) maximum rate of rise of left-ventricular pressure (dP/dt$_{max}$); (F) maximum rate of fall of LV pressure (dP/dt$_{min}$) and (F) time constant of LV pressure decay (Tau-W) at an intraventricular volume of 120 µl. Measurements were performed after 1.5 hours of reperfusion. *p<0.05 versus Control, #p<0.05 versus BD.

Figure 4. Histopathological and expressional analysis of inflammation and necrosis in the graft hearts after transplantation

Representative photomicrographs of hematoxylin-eosin stained sections (A-C) (magnification x100; scale bar: 100 μm) along with the semiquantitative scoring of myocardial (D) inflammation, (E) necrosis.

Quantitative real-time PCR analysis of myocardial mRNA levels of (F) Nuclear Factor (NF)-kappa β, (G) Tumor Necrosis Factor (TNF)-α. *$p < 0.05$ versus Control, #$p < 0.05$ versus BD.

Figure 5. DNA-strand breaks and expression of apoptotic factors in graft hearts after transplantation

The rate of TUNEL positive cells in each experimental group (A), quantitative real-time PCR analysis of myocardial mRNA levels of (B) caspase-3, (C) B-cell CLL/lymphoma 2 (Bcl-2), and densitometric analysis of bands for (D) cleaved caspase 3. *$p < 0.05$ versus Control, #$p < 0.05$ versus BD.

Representative photomicrograms of cardiomyocytes stained with (E) 4', 6-diamino-2-phenylindole (DAPI, blue), (F) nuclei with fragmented DNA visualized by TUNEL staining (red), and (G) merged image (magnification x400; scale bar: 20 μm).

Figure 6. mRNA and protein expression analysis of the HIF pathway in the graft hearts after transplantation

Quantitative real-time PCR analysis of myocardial mRNA levels (above) and densitometric analysis of bands (below) for (A-B) Hypoxia Inducible Factor (HIF)-1 alpha, (C-D) Heme-oxygenase (HO)-1, (E-F) Glucose Transporter (GLUT)-1, (G-H) Vascular Endothelial Growth Factor (VEGF). *$p < 0.05$ versus Control, #$p < 0.05$ versus BD.

21

Figure

Figure

Figure

Figure

Figure

Figure