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Brief Communication

Succinate Accumulation and Ischemia–Reperfusion Injury: Of Mice but Not Men, a Study in Renal Ischemia–Reperfusion

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A recent seminal paper implicated ischemia-related succinate accumulation followed by succinate-driven reactive oxygen species formation as a key driver of ischemia–reperfusion injury. Although the data show that the mechanism is universal for all organs tested (kidney, liver, heart, and brain), a remaining question is to what extent these observations in mice translate to humans. We showed in this study that succinate accumulation is not a universal event during ischemia and does not occur during renal graft procurement; in fact, tissue succinate content progressively decreased with increasing graft ischemia time ($p < 0.007$). Contrasting responses were also found with respect to mitochondrial susceptibility toward ischemia and reperfusion, with rodent mitochondria robustly resistant toward warm ischemia but human and pig mitochondria highly susceptible to warm ischemia ($p < 0.05$). These observations suggest that succinate-driven reactive oxygen formation does not occur in the context of kidney transplantation. Moreover, absent allantoin release from the reperfused grafts suggests minimal oxidative stress during clinical reperfusion.

Abbreviations: ADP, adenosine diphosphate; AUC, area under the curve; DCD, donation after cardiac death; HR-MAS, high-resolution magic-angle spinning; IR, ischemia–reperfusion; NMR, nuclear magnetic resonance; ROS, reactive oxygen species; SEM, standard error of the mean; TSP-d4, trimethylsilyl-tetradeuteropropionic acid; UPLC, ultraperformance liquid chromatography; WIT, warm ischemia time

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Introduction

Ischemia–reperfusion (IR) injury is the primary cause of organ dysfunction after events such as myocardial infarction, cerebrovascular accident and organ transplantation (1). Despite decades of intensive research and an abundance of promising preclinical results, no interventions emerged that prevent or reduce clinical IR injury (2,3). A recent study in mice proposed a new mechanistic concept (4): that progressive succinate accumulation during ischemia drives excessive postreperfusion reactive oxygen species (ROS) formation as a result of reverse electron transport through mitochondrial complex I. This excess ROS formation is suggested to drive IR injury (4).

Despite a wealth of rodent studies in support of a role for ROS in IR injury (5,6), human studies consistently fail to show a benefit with ROS scavenging (antioxidant) therapy (7–9). Moreover, although clinical studies confirm increased redox stress during IR, this is not followed by biomarkers of oxidative damage (10). Consequently, a pivotal role for oxidative damage as a driving force of IR injury (4) and thus a role for succinate accumulation is unclear for clinical IR injury. In this respect, it was noted that the proposed central role for succinate accumulation necessarily requires confirmation in the human setting (11).

Materials and Methods

Patient groups

Local ethics committees approved all experiments. Renal biopsies of 24 human kidney transplants were taken after written informed consent (10). Patients were classified (six per group) based on duration of cold ischemia: short (2–6 h), intermediate (6–12 h) and prolonged (12–24 h). Nonischemic controls were biopsies taken from functioning living donor kidneys. Renal allograft transplantations were performed according to the local standardized protocol. All grafts were preserved by static cold storage. The immunosuppressive regimen was based on induction therapy with basiliximab on days 0 and 4 and tacrolimus or cyclosporine A in addition to mycophenolate mofetil and steroids as maintenance therapy. Postoperative course was uneventful in all patients. One-year patient and graft survival was 100%. Patients in the short ischemia group did not develop clinical IR injury (delayed graft function), whereas 14% of the intermediate ischemia group and 71% of the prolonged ischemia group developed delayed graft function.

The influence of warm and cold ischemia on murine kidney succinate content was tested in kidneys from healthy surplus male mice (C57bl background). Kidneys were removed immediately on sacrificing. Reference (control) kidneys (n = 6) were directly snap frozen. Two series of six kidneys were kept on melting ice or in a warm-water bath (37°C) for 45 min to simulate cold and warm ischemia, respectively. Another six kidneys were kept on melting ice for 20 h before they were snap frozen to simulate prolonged cold ischemia.

Metabolic profiling

Metabolic profiling (hypoxanthine, succinate, and fumarate) of the tissue biopsies was performed by high-resolution magic-angle spinning (HR-MAS) nuclear magnetic resonance (NMR) spectroscopy. Human tissue samples were snap frozen in liquid nitrogen and stored at −80°C. Samples were prepared on ice and fitted in a leak-proof insert (30 μL, Kel-F; Bruker, Delft, Netherlands) used in a zirconium HR-MAS rotor (4 mm). The insert was filled with 3 μL cold (4°C) phosphate-buffered saline (in D_2O) containing 4.5 mM trimethylsilyl-tetradeuteropropionic acid (TSP-d4) and 25 mM sodium formate ($CHNaO_2$) as internal standards. NMR-spectrometry was used for murine kidney succinate content, for which metabolites were extracted and dried. The dried material was reconstituted with 0.3 mL phosphate buffer solution (pH 7.4; 150 mM K_2HPO_4; Sigma-Aldrich, Zwijndrecht, The Netherlands) in deuterated water containing also 0.2 mM of bacteriostatic NaN_3 and 0.4 mM sodium TSP-d4 (Cambridge Isotope Laboratories Inc., Buchem, Apeldoorn, The Netherlands) as chemical shift reference, and 0.19 mL of each sample was transferred to 3 mm NMR tubes (Bruker BioSpin, GmbH, Rheinstetten, Germany) for NMR analysis. HR-MAS spectra were recorded on a 14.1-T (600 MHz, 1H resonance frequency) Bruker Avance III NMR spectrometer equipped with a TCI cryoprobe. All experiments were acquired at 4°C while spinning the samples at a rate of 5 kHz. Metabolite signals were quantified using the BATMAN R package (12). Spectra were normalized based on the tissue weight used.

High-resolution respirometry

Effect of ischemia–reoxygenation on mitochondrial efficacy was analyzed by high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbrück, Austria) and compared with different species: humans (n = 7), pigs (n = 10), rats (n = 6) and mice (n = 6). Kidney biopsies from a healthy kidney segment were taken immediately prior to clamping of the renal vasculature and stored on ice. Warm ischemia was simulated by maintaining biopsies in a warm-water bath (37°C) for 60 min.

On simulating warm ischemia, the tissue was washed and permeabilized in saponin for 30 min at 4°C. Subsequently, the tissue was washed in mitochondrial respiration solution (MiR05; Oroboros Instruments) containing 0.5 mM ethylene glycol tetraacetic acid, 3 mM magnesium chloride, 60 mM κ-lactobionate, 20 mM taurine, 10 mM potassium dihydrogen phosphate, 20 mM HEPES, 110 mM sucrose and 1 $g.L^{-1}$ fatty acid-free bovine serum albumin (pH 7.1). Tissue was then weighed and placed in the measurement chamber of the high-resolution respirometer and incubated at 37°C. To avoid oxygen diffusion limitation, oxygen concentration was increased to ≈400 μM and maintained at >270 μM throughout the experiment by adding pure oxygen.

The integrity of the outer mitochondrial membrane was tested by adding 10 μM cytochrome C; samples with a >15% increase in respiratory rate were excluded from further analysis. Leak respiration was assessed by adding the Krebs cycle intermediates sodium glutamate (10 mM), sodium malate (2 mM) and sodium pyruvate (5 mM). Adenosine diphosphate (ADP)–stimulated respiration was measured in 2.5 mM ADP. Maximal respiration, with simultaneous input of electrons through complexes I and II, was measured through addition of 10 mM succinate. Maximal uncoupled respiration was measured after stepwise addition of 0.01 μM carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone. Subsequently, complex I was blocked by rotenone (0.5 μM). Finally, antimycin A was added to inhibit complex III, and residual oxygen consumption was measured (nonmitochondrial respiration) and subtracted from all values. All measurements were performed simultaneously and subsequently averaged. Values were mass normalized and reported as pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$. The contribution of complex I function to the uncoupled maximal respiration was assessed by the relative decline of respiration after adding rotenone.

Allantoin release

Postreperfusion allantoin formation was quantified by establishing arteriovenous concentration differences with the transplanted kidney (Figure S1). For maximum sensitivity, we specifically selected patients that presented with manifest IR injury (delayed graft function). These patients were all participants in a previous study (10).

Prior to implantation of the graft, a 5-French umbilical vein catheter was positioned in the lumen of the renal vein through one of its side branches. At 30 s and 3, 5, 10, 20, and 30 min after reperfusion (i.e. moment of reperfusion t = 0), 10-mL blood aliquots were sampled. Paired arterial blood samples were obtained. The abdominal wall was closed about 45 min after reperfusion, and the end point of sampling was reached 30 min after reperfusion. Blood samples were collected in precooled containers and immediately placed on melting ice. The validity of the arteriovenous sampling method was validated earlier, measuring oxygen saturation (10).

Allantoin in plasma was measured using an ultraperformance liquid chromatography (UPLC)–tandem mass spectrometry method developed

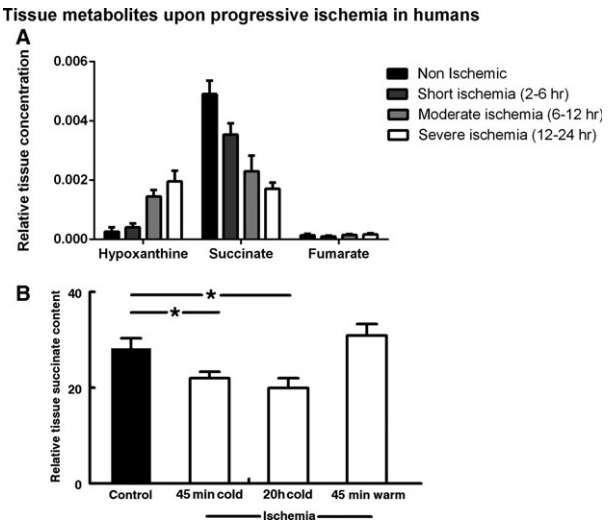

Figure 1: (A) Relative hypoxanthine, succinate, and fumarate tissue content (high-resolution magic-angle spinning nuclear magnetic resonance) on incremental ischemia time (human kidney). Increasing hypoxanthine ($p < 0.031$) but declining succinate levels ($p < 0.007$) during ischemia. Fumarate levels remained low and stable during ischemia. Nonischemic control values are included as a reference. All values were normalized on the basis of tissue weight (n = 6 per group, mean ± SEM). (B) Cold ischemia but not warm ischemia reduced murine kidney succinate content (*analysis of variance $p < 0.019$) (mean ± SEM). SEM, standard error of the mean.

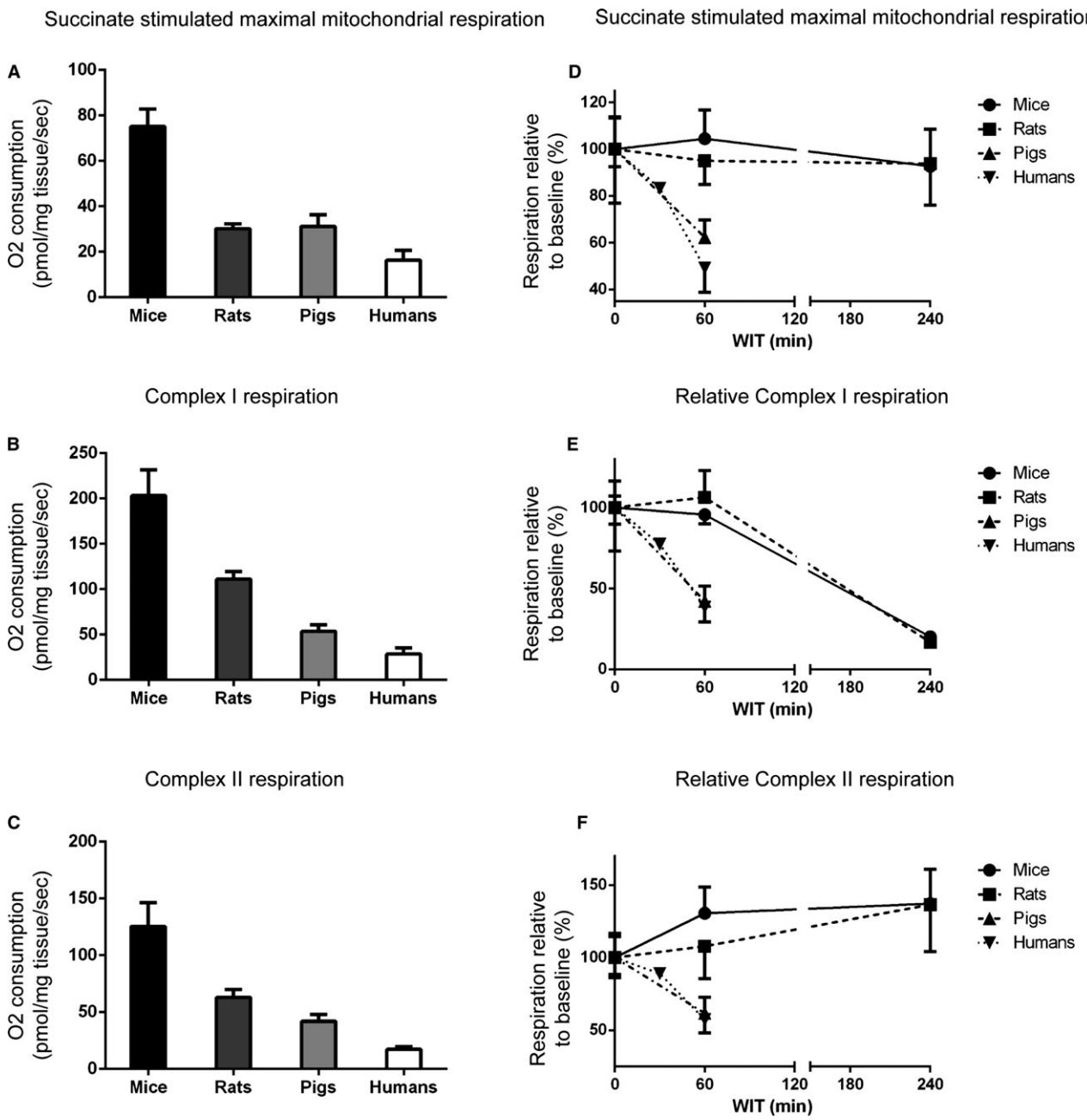

Figure 2: Interspecies differences in baseline mitochondrial respiration rates (A–C) and mitochondrial susceptibility toward ischemia and reperfusion (D–F). (A–C) Baseline aspects of mitochondrial oxidative phosphorylation (absolute values, oxygen consumption per mg of tissue per second) for different species. Note the inverse associations ($p < 0.000001$) between body weight and maximal oxidative phosphorylation capacity (A), complex I activity (B) and complex II activity (C). Bars represent mean ± SEM. (D–F) Effect of WIT and reoxygenation on the aspects of mitochondrial function for different species (values relative to baseline values [baseline = 100%], bars represent SEM). (D) Sixty minutes of warm ischemia significantly ($p < 0.05$) reduced succinate-driven mitochondrial respiration (combined complex I and II activities) in human and pig mitochondria, whereas succinate-driven respiration in rat and mouse mitochondria is not influenced by 4 h of warm ischemia. (E) Sixty minutes of warm ischemia significantly reduced complex I activity of human and pig mitochondria ($p < 0.03$). Complex I activity of mouse and rat mitochondria is not influenced by 60 min of warm ischemia, but significant drops are found after 4 h of warm ischemia ($p < 0.001$). (F) Sixty minutes of warm ischemia significantly reduced complex II (succinate dehydrogenase) activity of human and pig mitochondria ($p < 0.008$ resp. $p < 0.04$) but increased respectively stable complex II activity in mouse and rat mitochondria ($p < 0.038$). SEM, standard error of the mean; WIT, warm ischemia time.

in-house. Briefly 30 μL plasma was mixed with 30 μL of a solution containing stable isotope labeled internal standards. Samples were deproteinized with 500 μL acetonitrile. After centrifugation (10 min, 10 000 *g*, 4°C), the supernatant was evaporated under nitrogen and reconstituted in 500 μL 50 mM ammonium formiate (pH 4.00). Purine and pyrimidine metabolites were separated using a Waters Acquity UPLC system (Waters, Etten-Leur, Netherlands) equipped with an Acquity HSS T3 (2.1 × 100 mm, *df* 1.8 μm). Separation of the compounds of interest was achieved by a 0.01-M ammonium formiate (pH 4.00) and acetonitrile gradient. Compounds were quantified using a Waters XEVO TQS tandem mass spectrometer (Waters), with both negative and positive electrospray ionization using specific multiple reaction monitoring transitions.

Statistical analysis

Maximal mitochondrial respiration values were tested were using analysis of variance. Paired t-tests were used to compare the baseline respiratory rate with the respiratory rate after experiencing warm ischemia (SPSS 22.0; IBM Corp, Amsterdam, The Netherlands).

For the plasma allantoin measurements, the area under the curve (AUC) was estimated and compared through a linear mixed-model analysis for arterial and venous measurements for the total of 30 min. The model contained the following independent variables: time as categorical, the group, and the interaction between group and time. The covariance model was specified as unstructured. The ΔAUC was calculated (venous minus arterial), and the null hypothesis (AUC = 0) was tested by a Wald test based on the estimated parameters of the linear mixed model.

The level of significance was set at $p < 0.05$. All data represent mean plus or minus standard error of the mean.

Results

Similar to the report for the murine setting (which also specifically tested kidney IR) (4), our data for the human kidney show progressive hypoxanthine accumulation during ischemia (Figure 1). Results for succinate (and fumarate) contrast with those reported for the mouse (4). Fumarate concentrations remained low and stable during ischemia, whereas our clinical data showed a paradoxical decline in tissue succinate content following progressive ischemia (Figure 1A), indicating that succinate accumulation (4) is not a universal metabolic signature of ischemia. This contrast between the reported murine data (4) and our human data may reflect species-specific differences and relate in part to contrasting effects of warm and cold ischemia. We tested the latter by exposing murine kidneys to cold and warm ischemia. Figure 1B shows that although cold ischemia resulted in progressive but moderate succinate exhaustion ($p < 0.019$), tissue succinate levels remained stable during warm ischemia.

Interspecies differences also exist with regard to mitochondrial respiratory efficiency and ROS production (13). This is further illustrated by the clear reciprocal association between species body size and overall mitochondrial respiration (Figure 2A) and normalized activities of mitochondrial complex I and II activity (Figures 2B and C).

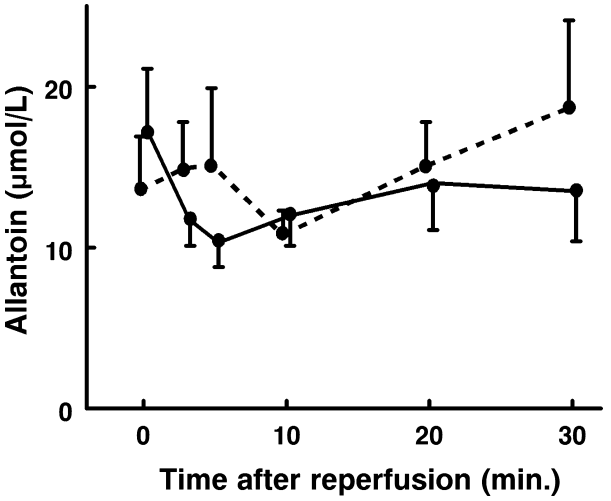

Figure 3: Absent postreperfusion uric acid reactive oxygen species quenching (allantoin release from the reperfused grafts following prolonged ischemia). Dotted line represents arterial allantoin levels, and solid line represents renal vein allantoin levels (arteriovenous difference by area under the curve, $p = 0.368$).

We tested for potential interspecies differences with regard to mitochondrial susceptibility to IR (Figures 2D–F), in particular with respect to succinate metabolism. Figure 2D shows that although postischemic mouse mitochondria retained or even increased their ability to oxidize succinate, reperfused human mitochondria lost their ability to oxidize succinate. Results from validation experiments using rat and pig renal mitochondria paralleled those for mice and humans, respectively (Figure 2D). In contrast to their rodent counterparts, postischemic human and pig mitochondria have a reduced capacity to oxidize succinate.

Respirometry revealed further interspecies differences in mitochondrial susceptibility. Not only are rodent mitochondria significantly more resistant to ischemia than human and pig mitochondria ($p < 0.05$ and $p < 0.03$, respectively) (Figures 2D and E), but it was observed that increases in complex II activity in rodent mitochondria compensate for loss of complex I function ($p < 0.038$) (14) (Figure 2F).

These findings challenge a prominent role for succinate-driven ROS formation in the context of renal graft IR (4). To that end, we tested reperfusion-related ROS formation in the context of clinical graft reperfusion by quantifying allantoin release from reperfused kidneys by means of arteriovenous concentration differences (Figure 3). Absent allantoin release in the first 30 min following reperfusion implies negligible ROS quenching during reperfusion.

Discussion

From these data, the picture emerges of divergent metabolic and mitochondrial responses to IR in mice and in humans and for succinate accumulation in murine kidneys between warm and cold ischemia. It is unclear how these observations translate to the human context. Prereperfusion succinate content of grafts from donation after cardiac death (DCD) was actually lower than that in grafts from donation after brain death (Figure S2), showing that the additional period of warm ischemia in DCD donors does not translate to increased graft succinate content.

In mice, progressive succinate accumulation during ischemia and reversed transport through mitochondrial complex I drives reperfusion-related ROS formation (4). In contrast, the human context is characterized by an inverse relationship between ischemia and succinate tissue levels and ischemia-driven knockdown of mitochondrial respiration. As such, the question arises of whether clinical IR in the context of kidney transplantation is accompanied by excess ROS formation.

As noted earlier, clinical studies consistently fail to show a beneficial effect of antioxidant therapy on IR injury (7–9), and markers for oxidative damage remain low following IR (10). These observations would be consistent with a minor role for ROS in clinical IR, but they also may indicate superior antioxidant responses, with effective ROS quenching, effectively preventing ROS-mediated damage. To test whether excess ROS is formed during clinical IR, we quantified ROS quenching by establishing allantoin release from reperfused human kidneys. Allantoin is the stable end product of ROS quenching by the antioxidant uric acid and, as such, reflects the extent of oxidative stress. Absent allantoin release in the first 30 min following reperfusion implies that ROS quenching is negligible and that ROS stress during clinical IR may be less than generally expected.

In summarizing the mechanism of succinate accumulation driving IR injury, Heger et al (11) pointed out that "most research in IR injury has been proven of limited translational value." The findings noted for IR in the clinical setting of kidney transplantation point to fundamental mechanistic differences between findings obtained in rodent models of IR and the actual human context. It came to our attention that these observations (partly) explain the apparent antioxidant paradox in the context of IR, with clear advantages in murine studies but no apparent benefit in clinical studies (7–9,15).

Disclosure

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

References

1. Hausenloy DJ, Yellon DM. Myocardial ischemia-reperfusion injury: A neglected therapeutic target. J Clin Invest 2013; 123: 92–100.
2. Cavaille-Coll M, Bala S, Velidedeoglu E, et al. Summary of FDA workshop on ischemia reperfusion injury in kidney transplantation. Am J Transplant 2013; 13: 1134–1148.
3. Lefer DJ, Bolli R. Development of an NIH consortium for preclinicAl AssESsment of CARdioprotective therapies (CAESAR): A paradigm shift in studies of infarct size limitation. J Cardiovasc Pharmacol Ther 2011; 16: 332–339.
4. Chouchani ET, Pell VR, Gaude E, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature 2014; 515: 431–435.
5. Marczin N, El-Habashi N, Hoare GS, Bundy RE, Yacoub M. Antioxidants in myocardial ischemia-reperfusion injury: Therapeutic potential and basic mechanisms. Arch Biochem Biophys 2003; 420: 222–236.
6. Okazaki T, Otani H, Shimazu T, Yoshioka K, Fujita M, Iwasaka T. Ascorbic acid and N-acetyl cysteine prevent uncoupling of nitric oxide synthase and increase tolerance to ischemia/reperfusion injury in diabetic rat heart. Free Radic Res 2011; 45: 1173–1183.
7. Firuzi O, Miri R, Tavakkoli M, Saso L. Antioxidant therapy: Current status and future prospects. Curr Med Chem 2011; 18: 3871–3888.
8. Myung SK, Ju W, Cho B, et al. Efficacy of vitamin and antioxidant supplements in prevention of cardiovascular disease: Systematic review and meta-analysis of randomised controlled trials. BMJ 2013; 346: f10.
9. Suzuki K. Anti-oxidants for therapeutic use: Why are only a few drugs in clinical use? Adv Drug Deliv Rev 2009; 61: 287–289.
10. de Vries DK, Kortekaas KA, Tsikas D, et al. Oxidative damage in clinical ischemia/reperfusion injury: A reappraisal. Antioxid Redox Signal 2013; 19: 535–545.
11. Heger M, Reiniers MJ, van Golen RF. Mitochondrial metabolomics unravel the primordial trigger of ischemia/reperfusion injury. Gastroenterology 2015; 148: 1071–1073.
12. Hao J, Liebeke M, Astle W, De IM, Bundy JG, Ebbels TM. Bayesian deconvolution and quantification of metabolites in complex 1D NMR spectra using BATMAN. Nat Protoc 2014; 9: 1416–1427.
13. Shi Y, Buffenstein R, Pulliam DA, Van RH. Comparative studies of oxidative stress and mitochondrial function in aging. Integr Comp Biol 2010; 50: 869–879.
14. Lapuente-Brun E, Moreno-Loshuertos R, Acin-Perez R, et al. Supercomplex assembly determines electron flux in the mitochondrial electron transport chain. Science 2013; 340: 1567–1570.
15. Braunersreuther V, Jaquet V. Reactive oxygen species in myocardial reperfusion injury: From physiopathology to therapeutic approaches. Curr Pharm Biotechnol 2012; 13: 97–114.

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: Arteriovenous blood sampling over the reperfused kidney. Arterial (red) venous (blue) concentration

measurements were measured against the reperfused kidney. Arterial blood was sampled through cannulation of the epigastric artery and the effluent; venous renal blood was sampled through cannulation of the renal vein via one of its side branches (gonadal vein).

Figure S2: Baseline succinate levels in kidneys from donation after brain death (cold ischemia only) vs. donation after cardiac death (warm and cold ischemia; p = 0.021). Bars represent mean plus of minus standard error of the mean.