Hypothermic oxygenated perfusion protects from mitochondrial injury before liver transplantation

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Abstract

Background: Mitochondrial succinate accumulation has been suggested as key event for ischemia reperfusion injury in mice. No specific data are however available on behavior of liver mitochondria during ex situ machine perfusion in clinical transplant models.

Methods: We investigated mitochondrial metabolism of isolated perfused rat livers before transplantation. Livers were exposed to warm and cold ischemia to simulate donation after circulatory death (DCD) and organ transport. Subsequently, livers were perfused with oxygenated Belzer-MPS for 1h, at hypothermic or normothermic conditions. Various experiments were performed with supplemented succinate and/or mitochondrial inhibitors. The perfusate, liver tissues, and isolated mitochondria were analyzed by mass-spectroscopy and fluorimetry. Additionally, rat DCD livers were transplanted after 1h hypothermic or normothermic oxygenated perfusion. In parallel, perfusate samples were analysed during HOPE-treatment of human DCD livers before transplantation.

Findings: Succinate exposure during rat liver perfusion triggered a dose-dependent release of mitochondrial Flavin-Mononucleotide (FMN) and NADH in perfusates under normothermic conditions. In contrast, perfusate FMN was 3-8 fold lower under hypothermic conditions, suggesting less mitochondrial injury during cold re-oxygenation compared to normothermic conditions. HOPE-treatment induced a mitochondrial reprogramming with uploading of the nucleotide pool and effective succinate metabolism. This resulted in a clear superiority after liver transplantation compared to normothermic perfusion. Finally, the degree of mitochondrial injury during HOPE of human DCD livers, quantified by perfusate FMN and NADH, was predictive for liver function.

Interpretation: Mitochondrial injury determines outcome of transplanted rodent and human livers. Hypothermic oxygenated perfusion improves mitochondrial function, and allows viability assessment of liver grafts before implantation.

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Abbreviations: AMP, Adenosine monophosphate; ADP, Adenosine diphosphate; ALT, Alanine aminotransferase; AS, Anastomotic structures; AST, Aspartate aminotransferase; ATP, adenosine triphosphate; CD-68, Cluster of differentiation 68; CS, cold storage; DAMPs, DANGER-associated molecular pattern’s; DBD, Donation after brain death; D-HOPE, Dual hypothermic oxygenated perfusion; DAMP’s, Damage associated molecular pattern’s; DBQ, Decylubiquinone; DC, Donation after circulatory death; DCD, Donation after circulatory death; D-HOPE, Dual hypothermic oxygenated perfusion; D-KMP, Donor warm ischemia time; EAD, Early allograft dysfunction; EC, Extracorporeal vessel oxygenation; ECMO, Extracorporeal membrane oxygenation; FAD, Flavin adenine dinucleotide; FMN, Flavin mononucleotide; GSH, Glutathione; HA, Hepatic artery; HAR, Hexammineruthenium; HAT, Hepatic artery thrombosis; HMGB-1, High mobility group box-1 protein; HMP, Hypothermic machine perfusion; HOPE, Hypothermic oxygenated perfusion; H&E, Hematoxylin and eosin; IC, Ischemic cholangiopathy; ICAM-1, Intercellular adhesion molecule-1; ICI, Intensive care unit; IMP, Inosine monophosphate; KC’s, Kupffer cells; LDH, Lactate dehydrogenase; LT, Liver transplantation; MELD, Model of end stage liver disease; MCT, Mitochondria permeability transition pore; MPT, Mitochondria permeability transition pore; NAD/NADH, Nicotinic adenine dinucleotide (oxidized/reduced); NAS, Non-anastomotic strictures; NBP, Normothermic regional perfusion; NMP, Normothermic machine perfusion; OAA, Oxaloacetate; OLT, Orthotopic liver transplantation; PNF, Primary non function; PV, Portal vein; RET, Reverse electron flow; ROS, Reactive oxygen species; SDH, Succinate dehydrogenase; SEC, Sinusoidal endothelial cells; TLR-4, Toll-like-receptor-4; 8-OHdG, 8-hydroxy-2-deoxyguanosine

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Research in Context

Evidence before this study

Recent work has highlighted that succinate accumulation during ischemia is an inevitable part of every organ procurement and transplantation, causing relevant injury during ischemia-reperfusion by electron overflow at mitochondrial complex I. While this scenario is well defined for re-oxygenation under normothermic conditions, little is known on mitochondrial behavior during cold oxygenation. Here we uncover in a translational project a novel mechanism, by which oxygenated reperfusion in the cold leads to significantly less mitochondrial injury in livers, despite succinate provocation, when compared to normothermic conditions.

Added value of this study

First, we show in rodent livers, that the release of mitochondrial flavin mononucleotide (FMN) during reoxygenation after ischemia under normothermic conditions depends on mitochondrial succinate. Secondly, reperfusion (reoxygenation) under cold conditions (HOPE) induces also mitochondrial FMN release, dependent on succinate levels, but to a significant less degree. Third, HOPE-treatment (cold oxygenation) induces a metabolic switch in mitochondria through an improved enzymatic activity of complex I, II and IV. Such findings correlate with a decrease of lactate, succinate, and purine derivates in liver tissues after HOPE, and are further paralleled by an increase of mitochondrial ATP and a reduced NADH/NAD ratio. Fourth, HOPE treated livers disclose consecutively less oxidative injury and less downstream inflammation during subsequent transplantation. Finally, the extent of mitochondrial FMN and NADH release during HOPE in human livers, and the linked nucleotide breakdown as a consequence of mitochondrial function, is a signature for liver graft injury, and serves as a predictor for human liver graft function before implantation.

Implications of the available evidence

We report a novel mechanism of preventing liver ischemia reperfusion injury, which allows simultaneously monitoring of liver graft injury while restoring mitochondrial function. We expect a high clinical impact due to the urgent clinical need for safe utilization of injured livers.

Introduction

Current discoveries in the field of hepatic ischemia-reperfusion (I/R) point to mitochondrial-derived oxidative stress as key mechanism of tissue damage [1–3]. Optimization strategies for the treatment of ischemic organs may, therefore, depend on effective protection from mitochondrial injury. Ex situ perfusion of organs for transplantation is a new and promising strategy to modify and improve cellular conditions before implantation. Two main perfusion methods for livers and kidneys have been developed and are currently under discussion in clinical practice [4]. The first approach aims to simulate the in vivo conditions of organ perfusion, at normothermic temperatures with blood and nutrients at physiological perfusion pressure and flow [5]. In contrast, an alternative technique involves hypothermic organ perfusion (4–12°C), with a blood-free preservation solution at non-physiologic perfusion pressure and flows [6]. The effect of both perfusion techniques on mitochondrial recovery of ischemic livers is currently unknown.

Here, we unravel the role of the respiratory electron chain during ex situ perfusion of ischemic livers under cold and warm conditions. We used a standardized ex situ rat liver perfusion model with synchronous fluorometric assessment of newly discovered markers of mitochondrial injury. We performed a metabolic analysis of liver tissues, intact mitochondria and acellular perfusate to assess mitochondrial function.

Collectively, our data suggest that, unlike normothermic oxygenated perfusion, hypothermic oxygenated perfusion is protective against liver ischemia-reperfusion injury. This phenomenon is triggered by a unique response of mitochondria to ischemia-reperfusion under hypothermic conditions. We have translated our findings in the human setting of liver transplantation, and developed a technique to monitor liver function by a combination of two metabolic markers, both detectable during cold perfusion and enabling us to evaluate the quality of donor livers and predict graft implantation success already before transplantation.

Methods

I. Rat liver experiments

Animals

Male Brown Norway rats (250–320 g) were used for all rat experiments. Animals were maintained on laboratory diet and water according to the Swiss Animal Health Care law. All experiments were approved by the cantonal veterinary of the University Hospital Zurich and the Centro di Stabulazione degli animali da laboratorio (Ce.S.A.L.), Firenze.

Study design and experimental groups

All experiments were carried out with a donation after circulatory death (DCD) animal model (30 min in situ warm ischemia) with subsequent cold ischemia for 4 h. Ex situ reperfusion was performed for 1h either normothermically (normothermic oxygenated perfusion, NT) at 37°C with Belzer MPS, or hypothermically (hypothermic oxygenated perfusion, HOPE) at 10°C with Belzer MPS. We added succinate every 10 min by repeated bolus injection, in the presence or absence of mitochondrial inhibitors during perfusion. We chose the following experimental groups with random assignment for NT or HOPE:

1. Belzer MPS without succinate
2. Belzer MPS + 2.5 mM succinate
3. Belzer MPS + 25 mM succinate
4. Belzer MPS + 250 mM succinate

Experiment 2 was repeated with either additional malonate (20 mM), rotenone (0.035 mM), or cyanide (20 mM) in the perfusate. Additional experiments were performed with adding ethanol (200 mM) in the presence or absence of 4-methylpyrazol (100 mM) during HOPE.

Additional experiments were also performed for NT with perfusion with diluted blood, and with NT performed after HOPE. In some experiments we performed also an intermittent perfusion stop (ischemia) for 10 min with subsequent reperfusion.

In a next step, we assessed metabolic and mitochondrial function in DCD rat livers before and after HOPE, without adding succinate during perfusion.
The following groups were investigated:

- Baseline: fresh resected liver without warm ischemia and minimal (0.5 h) cold ischemia
- DCD + CS: 30 min in situ warm ischemia + 4 h cold storage
- DCD + CS + 1 h HOPE: 30 min in situ warm ischemia + 4 h cold storage + 1 h HOPE

All experimental groups consisted of at least 6 experiments per group.

Liver procurement and cold storage

Following midline incision, cardiac arrest was induced by opening of the diaphragm without previous heparinization of donor animals. After 30 min of in situ warm ischemia, livers were flushed in situ with 10 ml cold heparinized Institute George Lopez (IGL)-1 solution via the portal vein. Livers were then excised (weight 9.8 ± 0.6 g) and placed in precooled IGL-1 solution at 4°C. Afterwards, livers tissues were either exposed to 4 h cold storage alone (for tissue analysis and mitochondrial isolation) or to 4 h cold storage followed by ex situ perfusion under different conditions (see above). For machine perfusion, all livers received a stent in the portal vein.

Normothermic oxygenated perfusion (HOPE)

Livers to be perfused at 10°C were connected to the precooled perfusion device after initial cold storage. Machine perfusion was maintained for 1 h under computerized pressure control (<3 mmHg), resulting in flow rates between 1 and 2 ml/minutes. Active oxygenation was provided by a hollow fiber oxygenator (Hugo Sachs Electronic), delivering a perfusate pO2 of 60–80 kPa. We used 100 ml recirculating UW gluconate solution (Belzer MPS) as perfusate. The perfusion box and perfusate were both maintained at 10°C by an open bath thermostat (Huber, Germany).

Liver transplantation

We performed orthotopic liver transplantation in controls (non-ischemic livers, baseline), cold storage DCD livers (30 min in situ warm ischemia + 4 h cold storage), in HOPE treated DCD livers (30 min in situ warm ischemia + 4 h cold storage + 1 h HOPE), and in normothermically perfused DCD livers (30 min in situ warm ischemia + 4 h cold storage + 1 h normothermically perfusion with diluted blood). Liver transplantation was performed as previously reported [7]. Liver tissue and blood samples were taken after 24 h.

Endpoints

1. Quantification of FMN

Real time release of flavin mononucleotide (FMN) in the perfusate was determined by fluorescence spectroscopy. A light probe was connected to a halogen light source to emit monochrome light at a wavelength of 450 nm on the recirculating perfusate (Fig. 1A-B). A spectrometric receiver probe with sufficiently high resolution (e.g. 4.6 nm) was used to quantify the proportion of emitted fluorescent light by the FMN molecules. The fluorescence emission maximum of FMN was measured between 500–600 nm (Avaspec HS2048XL-EVO, Avantes).

Flavin mononucleotide (FMN) was also determined by a lab-based fluorescence assessment from perfusate samples. All samples containing blood cells were primarily centrifugated before assessment. Additionally, we confirmed these measurements by targeted liquid chromatography-mass spectroscopy (LC-MS). Results from different types of measurements (fluorescence spectroscopy and LC-MS) were correlated for each sample. Fluorescence measurements were calibrated with standards to confirm increasing concentrations correlate with increasing positivity at the respective wavelength.

2. Quantification of NADH

Mitochondrial respiration rate during cold machine perfusion was measured by the amount of oxidized NADH (NAD+) determined spectroscopically and also by fluorescence in perfusates samples (excitation wavelength 360 nm, emission peak 460 nm). In addition, perfusate NAD+ and NADH values were determined by targeted Liquid Chromatography-mass spectroscopy (LC-MS). Results from different types of measurements (fluorescence spectroscopy and LC-MS) were correlated. The spectroscopy was calibrated with NADH standards to confirm increasing concentrations correlate with increasing positivity at the respective wavelength.

3. Quantification of 8-OHdG and HMGB-1 (in Perfusion)

Oxidative damage of DNA was detected in plasma by 8-hydroxy-2-deoxy Guanosine (8-OHdG) ELISA (Abnova; KA0444) and nuclear injury was measured by release of high mobility group box protein – 1 (HMGB-1; IBL International GmbH; STS1011).

4. Histology and Immunohistochemistry

The following staining procedures were performed after 1h perfusion in each experimental group: 8-OHdG (AA10051.1), Haematoxylin-Eosin (H&E) - staining for necrosis, Toll-Like-Receptor-4 -staining (TLR-4; LS-B2070, RRID:AB_1277478), CD 68 – staining (macrophilin; MCA341R, RRID:AB_2291300), ICAM (Santa Cruz, RRID: AB_631159). Histological assessments were determined by manual counting in 20 random visual fields (2.5x or 10x). All histological analyses were performed in a blinded fashion with respect to the experimental groups.

5. Metabolite extraction and profiling by targeted liquid chromatography-mass spectrometry (LC-MS/MS)

About 30 mg of N2 shock frozen liver tissues and 30 μl of perfusates samples (per experimental group and run) were used for metabolite profiling. Homogenization for liver tissues was performed with a FastPrep (60 s, 4 m x s⁻¹) in a buffer containing 1.5 ml methanol and 0.1% ammonium acetate. The protein pellet gained after brief centrifugation was used in a bicinchoninic acid (BSA) based protein assay (weight 9.8 ± 0.6 g). 0.5 ml of the protein pellet was used for targeted liquid chromatography-mass spectrometry (LC-MS/MS). Results from different types of measurements (fluorescence spectroscopy and LC-MS) were correlated for each sample. Fluorescence measurements were calibrated with standards to confirm increasing concentrations correlate with increasing positivity at the respective wavelength.

For metabolite extraction in perfusates, proteins were precipitated by the addition of two volumes of acetonitrile, vortexing for 5 min and centrifugation (16,000 g for 10 min. Again, the
protein pellets were used for normalization and an (IS) was added, as above. Metabolites in the supernatants were extracted with methyl tert-butyl ether (MTBE), methanol, and water [8].

Subsequent separation of the metabolites was performed on an LC instrument (1290 series UHPLC; Agilent, Santa Clara, CA), online coupled to a triple quadrupole hybrid ion trap mass spectrometer QTrap 6500 (Sciex, Foster City, CA), based on multiple reaction monitoring (MRM) method, as reported previously [9]. Three transitions per selected metabolite were measured.

Relative quantification was performed using MultiQuant™ software v.2.1.1 (Sciex, Foster City, CA). Peak integrations were reviewed manually and peak intensities were normalized, first against the internal standards, and subsequently against protein abundances obtained from the BCA assay. The first transition of each metabolite was used for relative quantification between samples.

Nicotine adenine dinucleotide (NAD), succinic acid, inosine, inosine monophosphate (IMP), adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), hypoxanthine, xanthine, fumaric acid, lactate, uric acid, and flavin mononucleotide (FMN) were determined by targeted LC-MS/MS.

6. Mitochondrial analysis, complex I, II, IV activity

Liver mitochondria membranes were isolated from fresh rat liver samples, e.g. DCD livers before and after HOPE or NT, immediately frozen in liquid nitrogen at -80°C. Healthy control livers, without exposure to ischemia, served as baseline. We performed, first, a metabolic analysis of isolated mitochondria, to determine purine, pyrimidine nucleotides and FMN in relation to mitochondrial succinate. Next, we investigated mitochondrial function of complex I, II, and IV as explained in detail in [24]. Complex I NADH-dependent activities were measured spectrophotometrically using Molecular Device plate reader (Varian Cary 4000 or plate-reader Molecular Devices) at 25°C as a decrease in absorption at 340 nm with 120 μM NADH in standard assay medium (0.25 M sucrose, 50 mM Tris-HCl pH 8.5, 0.2 mM EDTA, 15 μg/ml alamethicin, 2 mM MgCl₂) and containing 15–20 μg protein/ml of membranes. Permeabilization with alamethicin stimulates any NADH-dependent activities around 30%, indicating that our preparation contains 30% of still intact mitochondria membranes even after freezing/thawing cycle. In order to estimate relative content of Complex I, measurements of NADH: H⁺ oxidoreductase were carried out in the standard assay medium with 1 mM HAR. Rotenone sensitive (>95%) NADH: decylubiquinone reductase activity of Complex I alone was measured in standard assay medium supplemented with cyanide (2 mM) and 31 μM decylubiquinone (DBQ). Ferrocyanochrome c oxidase activity of Complex IV was measured in assay medium (125 mM KCl, 14 mM NaCl, 20 mM HEPES, 0.2 mM EGTA pH 7.2) with 0.025% n-dodecyl β-d-maltoside (DDM) and 55 μM of reduced ferrocyanochrome c [10]. The enzymatic activity was 100% sensitive to 0.5 mM cyanide. Succinate dehydrogenase activity was determined spectrophotometrically with Q1 (15 μM) as electron acceptor in standard medium supplemented with 20 mM succinate and 1 mM cyanide. All obtained mitochondrial preparations catalyze succinate oxidation (not shown) with a pronounced lag-phase with initial rate is 30–60% of the final linear steady-state rate (not shown). Prior to activity measurements membranes were incubated with malonate to replace OAA from the binding site (30 min × 30C).

II. Human liver perfusion

Perfusate samples were obtained during hypothermic oxygenated perfusion (HOPE) of human liver grafts donated after circulatory death (DCD). Following standard cold storage with IGL-1, livers underwent a pressure controlled hypothermic perfusion at 10°C with highly oxygenated perfusate (PO₂ 60–80 kPa) for 1–2 h. Perfusion was performed at 10°C using Belzer MPS (Bridge to Life) as perfusate and the Liver Assist device (Organ Assist). During perfusion, multiple
perfusate samples were obtained and analyzed by fluorimetry (perfusate 1:4 diluted) and LC-MS. The results were correlated to clinical parameters after liver transplantation, including graft survival. HOPE perfusates were also assessed for nicotine adenine dinucleotide (NAD), succinic acid, inosine, inosine monophosphate (IMP), hypoxanthine, xanthine, uric acid, N-acetyl-L-glutamic acid and flavin mononucleotide (FMN) through LC-MS.

Quantification and Statistical analysis

All activities were measured in duplicates or quadruplicates at 25° C. All activities are shown in μmol substrate/min/mg protein. All data are presented using the median and interquartile range (IQR) for continuous variables. The non-parametric Mann-Whitney U-test was used to determine whether significant differences existed between 2 groups. A p value of <0.05 was deemed statistically significant. Pearson’s parametric correlation was used to assess the relationship between to metric variables.

Receiver-operating characteristic (ROC) curves were used to determine the threshold of highest sensitivity and specificity in human perfusate FMN samples for liver graft loss.

All data were analyzed using IBM® SPSS® v.24.0 (Armonk, NY: IBM Corp.) and GraphPad Prism, version 8.0 (San Diego, CA, USA).

Quality control and ethical approval

Completeness, plausibility, and validity of the data were independently verified (by A.Sc., X.M., A.St., M.M., P.M., A.G., D.M. and P.D.). The study approval from the cantonal ethics commission was obtained prior to study initiation (ZH-Nr. 2015-0200). Animal experiments were approved by the cantonal veterinary office Zurich (01/2012, 218/2014) and protocol No 90/2020-PR.

Role of the Funding source

We confirm that all funders played no role in study design, data collection, data analysis, interpretation, or writing of the report.

Results

Mitochondrial response during normothermic and hypothermic liver reperfusion

Oxygenation of ischemic tissue results in oxidative stress, generated by an electron leak at mitochondrial complex I [1,11]. The primary driver of such mitochondrial-derived reactive oxygen species (ROS) is an ischemic accumulation of the tricarboxylic acid (TCA) cycle metabolite succinate [3,11–13]. Data obtained from ischemic brain mitochondria have further suggested, that the flavin mononucleotide (FMN) site of complex I is responsible for the production of ROS via reverse electron transfer (RET), and that accumulation of succinate triggers release of FMN from mitochondrial complex I [2,14,15].

We intended, therefore, to investigate, if mitochondrial FMN release occurs also in livers during I/R, and how this phenomenon depends on temperature. We used an isolated perfused rat liver model, with circulating acellular buffer (Belzer Machine Perfusion Solution (MPS)), the currently most used machine perfusate for all abdominal organs [16,17]. We chose a clinical related model with livers injured by a combination of warm in situ ischemia, simulating donation after circulatory death (DCD), and subsequent cold storage, simulating conventional organ transport before transplantation. Furthermore, we added succinate to the circulating perfusate and analyzed succinate uptake in liver mitochondria at different temperatures, as well as related mitochondrial injury.

For this purpose, we applied a continuous real-time fluorimetry of the perfusate, using an excitation wavelength at 360 nm (NADH) and 450 nm (FMN), and emission spectra between 400 and 600 nm (Fig. 1, Supplemental Figure 1d). High levels of perfusate oxygen (pO2 60–80 kPa) compensated the lack of red blood cells [18–20].

Normothermic reoxygenation of DCD livers causes mitochondrial injury

In a first step, we showed, that cumulative mitochondrial succinate uptake from perfusate was not different between hypo- and normothermic perfusion (Supplementary Fig. 1a). Due to limited cellular membrane permeability for succinate, however, mitochondrial succinate increase was approximately 200-fold less than added perfusate succinate (Supplementary Fig. 1b). Next, we demonstrated that acellular perfusion of rodent DCD livers at 37°C, without including mitochondrial substrates, resulted in a significant release of FMN in the perfusate (Fig. 2a), detectable by both, fluorimetry and NMR analysis (Rho = 0.8327) (Fig. 2b). Notably, perfusate FMN increased further under normothermic perfusion with blood instead of Belzer MPS (Fig. 2a). We observed also, that the presence of succinate in the circulating perfusate at normothermic (37°C) conditions, triggered a dose-dependent FMN increase (Fig. 2 c, d), similar as intermittent exposure of livers to 10 min ischemia (Fig. 2c, Supplementary Fig. 1d). Furthermore, perfusate FMN correlated well with perfusate NAD/H NADH and IMP (Fig. 2e, f), but not with the release of cytosolic enzymes, e.g. AST and ALT (Fig. 2f), indicating the absence of cell rupture. In addition, mitochondrial FMN and nucleotide content were inversely related to perfusate values (Fig. 2h, Supplementary Fig. 1c, e, f), pointing to mitochondrial origin of FMN release.

Next, high perfusate succinate concentrations under normothermic conditions induced oxidative stress, assessed by an increased concentration of 8-OHdG and HMGB in the perfusate, and with downstream activation of Kupffer cells, visualized by specific immunostaining for 8-OHdG, and toll-like receptor-4 activation (TLR-4) (Fig. 3a–d).

Third, we showed that succinate in the presence of malonate, a competitive inhibitor of the carboxylate binding site of complex II, increased once more NADH and FMN release (Fig. 4a, c), in contrast to inhibition of complex IV by cyanide or inhibition of complex I by rotenone (Fig. 4c). Succinate plus malonate resulted in increased purine salvage, detectable by high IMP simultaneously with low hypoxanthine and xanthine (Fig. 4e–h).

Hypothermic reoxygenation of DCD livers is less harmful

In another set of experiments, we investigated whether FMN released from mitochondria occurred also during hypothermic oxygenated perfusion (HOPE) at 10°C. However, in contrast to the normothermic perfusion experiments above, we observed a 3 to 8-fold lower FMN release with and without succinate exposure (Fig. 2g, 4d). Consistently, the NADH content in the perfusate was significantly less, compared to normothermic conditions, despite an excess of succinate during HOPE (Fig. 3b, 4b), and mitochondrial concentrations of FMN remained high (Fig. 2h). In addition, downstream activation of Kupffer and endothelial cells were not detectable during HOPE (Fig. 3), indicating less overall inflammation. Of note, neither inhibition of complex I, II or complex IV resulted in increased FMN (Fig. 4d). Increased mitochondrial injury was further visualized by staining of released NDUF5-1, e.g. one of the core subunits of the N-module of complex I, which accommodate FMN and the iron clusters N1a, N3, N1b, N4, and N5. Cytosolic NDUF5-1 was detectable after normothermic perfusion with succinate in contrast to hypothermic perfusion (Fig. 2b).

Importantly, while succinate alone failed to provoke high release of FMN under hypothermic oxygenated conditions, inhibition of complex I and II together caused FMN release (Fig. 4d), as well as adding
Fig. 2. a: Detection of mitochondrial FMN and metabolite release in perfusates: Release of FMN occurred at various perfusate conditions, including hypothermic or normothermic acellular perfusion, and by normothermic perfusion with whole blood, which resulted in highest FMN release (n = 20) (a). Perfusate FMN, detected by fluorescence, was validated by NMR analysis (Rho = 0.8327) (measured in all rodent samples n > 55) (b). Administration of succinate or intermittent ischemia/reperfusion triggered relevant FMN release during normothermic perfusion (n = 6–7 for all groups other than: 60 min normotherm alone: n = 19 and n = 4 in FMN calibration) (c). Release of FMN increased in accordance with the amount of administered succinate (2.5 mM < 25 mM < 250 mM succinate; n = 6–7 for all groups) (d). Perfusate NADH quantification through spectroscopy demonstrated increasing concentrations according to administered succinate (n = 15 for all groups other than: NT without succinate, n = 5) (e). Perfusate FMN concentrations correlated also well with NAD (Rho = 0.9746) and IMP (Rho = 0.8633), in contrast to release of cytosolic liver enzymes (AST, ALT) (n = 50, human samples) (f). Neither succinate application or intermittent ischemia/reperfusion was found to trigger high FMN release during hypothermic oxygenated perfusion (n = 6 other than calibration group, n = 4 and end of 60 min hypotherm: n = 18)
of NADH donors (ethanol). In contrast, adding ethanol in the presence of methylpyrazol, e.g. inhibiting the NADH producing ethanol dehydrogenase, prevented FMN release (Fig. 4d). Such experiments support the hypothesis, that FMN release is mainly induced in the cold by an increase of reduction state of complex I and increase of NADH/NAD+ ratio [2,21].

In summary, under conditions of normothermic reperfusion in the presence of high intra-mitochondrial succinate concentrations, complex I-bound FMN dissociates due to over-reduction of the enzyme. A reductive dissociation of FMNH2 would be followed by a rapid non-enzymatic oxidation to FMN by oxygen with ROS formation[22]. A consequence, mitochondrial oxidative injury occurs under normothermic reperfusion conditions, despite succinate provoke and despite the presence of malonate (Fig. 5a). In contrast, FMN release and oxidative stress decreases significantly under hypothermic reperfusion conditions, despite succinate administration and despite the presence of malonate (Fig. 5b). We conclude, that HOPE treatment for one hour in rat DCD livers is sufficient to induce mitochondrial reprogramming towards more oxidized state and to upload the adenine nucleotide pool. This effect is caused by the improved function of mitochondrial respiratory complexes.

Liver transplant function improved after HOPE compared to normothermic oxygenated perfusion

To further confirm our findings, we analyzed liver function, liver tissue ATP and histology after transplantation of unperfused and perfused DCD rat livers (30 min warm in situ ischemia + 4 h cold storage), e.g. after cold storage alone, after endischemic normothermic oxygenated perfusion with diluted blood for 1 h, and after endischemic

Metabolic reprogramming of ischemic rat liver mitochondria by HOPE treatment

After demonstrating low mitochondrial injury during cold reoxygenation of rat DCD livers, we analyzed mitochondrial function in isolated mitochondria after cold storage, and after applying HOPE subsequently to cold storage. We found, first, a clear decrease in both NADH: hexammineruthenium (HAR) reductase and NADH: decylubiquinone (DBQ) reductase activity of complex I in conventional stored DCD livers (Fig. 6a; a). In contrast, HOPE treatment of cold-stored DCD livers significantly improved the enzymatic activity of complex I (Fig. 6a; a). Secondly, HOPE also increased the activity of complex II and complex IV (Fig. 6a; b/c). These results correlated well with a significant decrease of lactate, succinate, and hypoxanthine levels in liver tissues by HOPE, as well as in an increase of liver ATP (Fig. 6a), and a significant decrease of the NADH/NAD ratio.

We confirmed these results in additional experiments on a mitochondrial level, with application of high doses (250 mM) of perfusate succinate during HOPE, followed by normothermic reperfusion. HOPE treatment enabled mitochondria to completely fuel down succinate and restore nucleotides, while preventing FMN loss and downstream inflammation, in contrast to untreated livers (Fig. 6b).
Mechanism of reoxygenation under normo- and hypothermic conditions: Under conditions of normothermic reperfusion in the presence of increased intra-mitochondrial succinate concentrations, complex I-bound FMN dissociates due to over-reduction of NADH dehydrogenase. A reductive dissociation of FMNH₂ would be followed by a rapid oxidation to FMN by oxygen with ROS formation and significant mitochondrial dysfunction, leading to ATP breakdown. As a consequence, mitochondrial oxidative injury occurs under normothermic reperfusion conditions, and is further aggravated by malonate. In contrast, FMN release and oxidative stress decreases significantly under hypothermic reperfusion conditions, despite succinate provoke and despite the presence of malonate.

Fig. 4. Assessment of mitochondrial function during normo- and hypothermic perfusion in the presence of mitochondrial inhibitors: Various mitochondrial inhibitors were administered during liver perfusion together with succinate. Inhibition of complex II by malonate triggered increase of perfusate NADH during normothermic (a) and during hypothermic conditions (b). However, complex II inhibition caused only under normothermic conditions relevant FMN release (c), in contrast to hypothermic perfusion (HOPE) (d). During HOPE, high FMN release occurred with a combination of complex I and complex II inhibitors, or by excess of NADH donors (d). Administration of increasing succinate, or administration of a combination of succinate and malonate, led to increasing NAD in perfusates under normothermic conditions (e). Succinate plus malonate resulted also in increased purine salvage, detectable by high IMP (f) simultaneously with low hypoxanthine and xanthine (n = 8 – 10, single measurements or duplicates) (g, h) (Mann-Whitney U-test was used for statistical analysis).

Fig. 5. Mechanism of reoxygenation under normo- and hypothermic conditions: Under conditions of normothermic reperfusion in the presence of increased intra-mitochondrial succinate concentrations, complex I-bound FMN dissociates due to over-reduction of NADH dehydrogenase. A reductive dissociation of FMNH₂ would be followed by a rapid oxidation to FMN by oxygen with ROS formation and significant mitochondrial dysfunction, leading to ATP breakdown. As a consequence, mitochondrial oxidative injury occurs under normothermic reperfusion conditions, and is further aggravated by malonate. In contrast, FMN release and oxidative stress decreases significantly under hypothermic reperfusion conditions, despite succinate provoke and despite the presence of malonate.
hypothermic oxygenated perfusion with Belzer MPS for 1 h. The results showed in accordance to earlier reports[7], that normothermic perfusion improved ATP and decreased hepatocellular injury compared to cold storage. HOPE treatment resulted however in further upload of ATP and best protection compared to cold storage or endischemic normothermic perfusion (Fig. 6c).

Flavin and NAD/NADH in liver perfusate serves as viability marker in human liver transplantation

Based on previous experimental research, we have been using cold oxygenated machine liver perfusion (HOPE) since 2012 in human extended criteria donor livers before transplantation, to optimize graft quality[16]. Currently, approximately 120 HOPE-treated human livers have been implanted in Zurich, with an overall 5-year graft survival, censored for tumor related recipient death, of more than 90%[16]. Within a translational aspect of the present study we analyzed the perfusate for FMN and metabolite release of fifty human livers, treated by HOPE before implantation. Corresponding to the experiments in rodent DCD livers above, we found an initial FMN release within the first 10–30 min of HOPE treatment in human livers (Fig. 7a), with significant differences comparing DCD and DBD grafts (Fig. 7b). In addition, the quantity of perfusate FMN during HOPE correlated well with graft function and clinical outcome after graft implantation. For example, perfusate analysis of livers, which developed irreversible graft injury, e.g. primary non-function (PNF) or irreversible cholangiopathy, revealed extremely high FMN values within the first 30 minutes of HOPE perfusion, which was therefore predictive for graft loss (c-statistic 0.94, Fig. 7c). A comparison of metabolites in perfusates of failed (n = 7) vs survived grafts (n = 43) showed 8-fold higher FMN values and 6-fold higher NAD values in grafts which were lost (Fig. 7d). All purine metabolites (IMP, inosine, xanthine, hypoxanthine, uric acid) were also significantly different.

We conclude, that the extent of nucleotide breakdown as a consequence of mitochondrial dysfunction is a signature for graft injury, and predictive for human liver graft function. FMN and NAD/NADH release appear as novel perfusate markers for assessing mitochondrial metabolism and cumulative graft injury already during donor liver machine perfusion.

Discussion

The results of this study point to a uniform mechanism of mitochondrial-based liver I/R injury. First, we show that succinate accumulation and flavin mononucleotide (FMN) release from
Fig. 6 Continued.
mitochondria are initial events during liver reperfusion, leading to a multifactorial injury including mitochondrial ROS release, DAMP signaling, and activation of non-parenchymal liver cells. Second, we demonstrate that exposure of mitochondria to a combination of succinate and oxygen leads to significantly less mitochondrial FMN dissociation under cold compared to normothermic conditions. Third, we show, that hypothermic oxygenation of mitochondria induces a metabolic reprogramming within one hour, with a significant decrease of succinate and uploading of the intracellular nucleotide content. Next, we translated our results to human and demonstrate that FMN release and NADH oxidation during HOPE serves as an easily detectable novel parameter to predict liver function prior to implantation.

Our data underline recent findings that accumulation and mitochondrial oxidation of succinate causes extensive damage in transplanted organs, and should therefore primarily be targeted by new therapeutic methods to improve outcome [23]. Mitochondrial complex I catalyzes the first step of the mitochondrial electron transfer in the respiratory chain. It contains 8 iron-sulfur clusters as well as non-covalently bound FMN, and is solely responsible for the oxidation of NADH, generated during catabolism (glycolysis, TCA cycle, etc.). It is also the major contributor to ATP synthesis in mitochondria [12]. Under physiologic conditions, FMN is tightly bound to the enzyme. However, FMN loss from complex I is possible when complex I is fully reduced, i.e. when the electron transport chain is halted during ischemia [15,16,24,25].

Recent reports have suggested, that mitochondrial RET can occur between complex II and I early upon reperfusion of ischemic tissue, due to ischemic accumulation of succinate [26–28]. In addition, adenine nucleotides are degraded during I/R, caused by progressive hydrolysis of ATP to AMP during ischemia, with a further breakdown by the purine nucleotide cycle [29,30], generating hypoxanthine and inosine. During normothermic reperfusion, the restoration of adenine nucleotide levels is delayed by approximately 45 min, due to the relatively slow rate of the purine salvage pathway, resulting in low levels of ADP during the early reperfusion phase [29,31,32]. Simultaneously, accumulated succinate is rapidly oxidized by complex II under conditions of ADP deficit, resulting in hyperpolarization of the membrane, and therefore supporting RET at complex I [33]. In steady-state conditions this leads to a complete reduction of the NAD+ pool and is also associated with the highest rate of ROS formation in the mitochondrial matrix [11,33].

As demonstrated in brain mitochondria, an over-reduction of complex I induce FMN dissociation from the enzyme, causing its inactivation [14,15,24]. A similar loss of mitochondrial FMN during ischemia has been described during cardiac I/R in a dog model [25]. Here we confirm, that the presence of succinate triggers significant mitochondrial FMN release and NADH accumulation in rat and human DCD livers during normothermic oxygenated perfusion, together with oxidative injury and downstream activation of toll-like receptors of non-parenchymal liver cells. However, FMN release was not preventable in our model by malonate and...
appeared therefore not RET-induced. We interpreted this as direct evidence for complex I damage with mitochondria operating preferably in Mode 1 according to Murphy [1], underlining an early oxidative injury of complex I, for example by modification of critical thiol residues (-SH) [34].

In contrast, oxygenation of liver tissue under hypothermic conditions protected mitochondria strongly from FMN release, despite excessive succinate load, while the addition of NADH donors provoked FMN release also during hypothermic oxygenation. In addition, the mitochondrial complex I and II activity even improved during 1 h HOPE.

Such remarkable differences in FMN release under hypothermic versus normothermic perfusion conditions is related to a unique mitochondrial response under cold conditions. A slow-down of the mitochondrial activity in the cold, e.g. by decreased proton motive force during hypothermic oxygenation after ischemia, or by delayed D-A transition of complex I may prevent over-reduction of FMN and subsequently lessen FMNH2 release. Based on this, the central protective mechanism of cold oxygenation after ischemia depend on effective oxidation of accumulated succinate while avoiding mitochondrial injury [35]. Likewise, protective effects of HOPE in rodent DCD hearts were clearly related to succinate metabolization [36].

Of note, a reversible downregulation of the electron transport has already been reported in all forms of mammalian hypometabolism during natural hibernation [37,38], as well as after pharmacological induced suspended animation [39]. We may speculate, that inducing a hypometabolic state before normothermic reperfusion is eventually related to an ancestral behavior of mitochondrial proteins in the cold, similarly to the protection from oxidative stress during arousal after torpor [38,40]. For example, arctic ground squirrels tolerate hypoxia during arousal without subsequent development of relevant ischemia-reperfusion injury. Likewise, during hibernation, arterial oxygen saturation is significantly higher compared to euthermia [41], and the ATP level is well preserved [42], similarly to hypothermic oxygenated perfusion [30,43–49]. These findings were paralleled by the determination of the key role of complex I-derived oxidative stress in aging comparing different species with a short or long life span.

A limitation of our model is the lack of a direct proof of FMN loss by mitochondrial complex I. However, based on the comparison of mitochondrial and perfusate FMN, which was inversely related, we indirectly concluded mitochondrial origin of FMN release. Notably, FMN sources in mammalian cells are limited, with only 6 out of 76 flavoproteins containing non-covalently bound FMN, which potentially can be released. Of those, only NADH dehydrogenase, e.g. complex I, is located intra-mitochondrial, thus underlining our hypothesis.

Another shortcoming is the use of un-physiologic high succinate concentrations in our perfusion model, e.g. 2.5, 25, 250 μM. However, perfusate succinate application was entirely pH-adjusted, and was used as a tool to induce a highly standardized increased level of mitochondrial succinate. Due to poor membrane permeability of succinate, the concentrations of perfusate succinate needed to be high enough for provoking more than 10 fold changes in mitochondrial succinate concentrations, which have been recently reported to occur under liver ischemia [3].

We believe that our results have a direct clinical impact because transferring organs into a hibernation-like status could be a novel protective concept [34,40]. This approach is however in contrast to an upfront normothermic liver perfusion strategy [50–54]. Our data demonstrate instead, that in situ and ex situ normothermic perfusion is likely to induce inflammatory pathways through mitochondrial-mediated injury, depending on the level of succinate accumulation, consistent with recent findings on ex situ normothermic perfusion of other solid organs, for example of ischemic hearts, lungs, and kidneys [55]. This injury occurs within the first few minutes of normothermic reperfusion and is unlikely to be completely preventable by inhibitors of RET. Instead, an effective upregulation of mitochondrial repair mechanism, as seen in vivo, would be required, implicating long-term normothermic perfusion strategies, e.g. perfusion for several days or weeks [56].

Importantly, mitochondrial injury can also occur during cold oxygenated perfusion, yet at much lower levels. The addition of mitochondria-targeted anti-oxidative scavengers to the machine perfusate would, therefore, be a further topic of research [57]. The unique figure of mitochondrial response at hypothermic temperatures allows, however, fueling of accumulated succinate during cold oxygenated perfusion, without significant injury to complex I. Simultaneously, the cellular ATP pool is uploaded by continuous fueling proton pumps and ATP synthase. Liver cells are therefore recharged with nucleotides without significant injury. Importantly, eventual complex I injury, as displayed by FMN- and NADH release, during hypothermic oxygenated perfusion can be quantified before using the graft for transplantation. This measurement is elegantly performed in a real-time fashion from perfusates obtained during machine liver perfusion. Remarkably, such a concept originates from an earlier publication 50 years ago on the fluorometric assessment of mitochondrial redox changes [58].

In summary, we demonstrate, that perfusion of the ischemic liver with a hypothermic oxygenated solution (HOPE) prevents the impairment of the mitochondrial electron transport chain and protects from subsequent reperfusion injury. This result is in stark contrast to reperfusion under normothermic conditions. Short-term, cold oxygenation for mitochondrial conditioning may, therefore, be considered for ischemic solid human organs prior to ex situ normothermic perfusion or implantation.

Contributors

Conceptualization, A.Sc. and P.D.; Methodology, A.Sc., X.M., A.St., M.M., A.G, D.M., and P.D.; Investigation, A.Sc., X.M., A.St., M.M., P.K., O. dR., P.M., A.G., D.M. and P.D.; Writing – Original Draft, A.Sc. and P.D.; Writing – Review & Editing, A.Sc., X.M., M.M., P.M., A.G., D.M. and P. D.; Funding Acquisition, A.Sc., A.G., D.M. and P.D.; Resources, P.M., A. G., D.M., and P.D.; Supervision, P.M., A.G. and P.D. All authors read and approved the final version of the manuscript.

Declaration of Interests

This study was conducted at the University Hospital Zurich (USZ). Raw data and laboratory analysis results were extracted at the laboratory of the Department of Visceral Surgery and Transplantation of the USZ. Data analysis was carried out at the laboratory of the Department of Visceral Surgery and Transplantation of the USZ. The authors have declared that no competing interests exist. The study was principally funded by a main research grant from the Swiss national foundation (SNF), awarded to Professor Philipp Dudkowski (Ref: 32003B-140776/1, 3200B-153012/1, 31IC30-166909). Mitochondrial and tissue analysis was further supported by the Max Planck Society (Dr. David Meierhofer) and the NIH grant: R01NS112381 awarded to Dr. Alexander Galkin. There are no patents involved or affecting this study.

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Data Sharing Statement

Will individual participant data be available (including data dictionaries)? Yes. What data in particular will be shared? Individual participant data that underlie the results reported in this article, after de-identification (text, figure 7, and supplemental material). What other documents will be available? None. When will data be available (start and end dates)? Beginning 1 year after publication until 3 years. With whom? Investigators whose proposed use of the data has been approved by an independent review committee (“learned intermediary”) identified for this purpose. For what types of analyses? To achieve aims in the approved proposal. By what mechanism will data be made available? Proposals may be submitted up to 36 months following article publication.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.103014.

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