Mitochondrial injury during normothermic regional perfusion (NRP) and hypothermic oxygenated perfusion (HOPE) in a rodent model of DCD liver transplantation

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Summary

Background Normothermic regional perfusion (NRP) and hypothermic-oxygenated-perfusion (HOPE), were both shown to improve outcomes after liver transplantation from donors after circulatory death (DCD). Comparative clinical and mechanistical studies are however lacking.

Methods A rodent model of NRP and HOPE, both in the donor, was developed. Following asystolic donor warm ischemia time (DWIT), the abdominal compartment was perfused either with a donor-blood-based-perfusate at 37 °C (NRP) or with oxygenated Belzer-MPS at 10 °C (donor-HOPE) for 2 h. Livers were then procured and underwent 5 h static cold storage (CS), followed by transplantation. Un-perfused and HOPE-treated DCD-livers (after CS) and healthy livers (DBD) with direct implantation after NRP served as controls. Endpoints included the entire spectrum of ischemia-reperfusion-injury.

Findings Healthy control livers (DBD) showed minimal signs of inflammation during 2 h NRP and achieved 100% posttransplant recipient survival. In contrast, DCD livers with 30 and 60 min DWIT suffered from greater mitochondrial injury and inflammation as measured by increased perfusate Lactate, FMN- and HMGB-1-levels with subsequent Toll-like-receptor activation during NRP. In contrast, donor-HOPE (instead of NRP) led to significantly less mitochondrial-complex-I-injury and inflammation. Results after donor-HOPE were comparable to ex-situ HOPE after CS. Most DCD-liver recipients survived when treated with one HOPE-technique (86%), compared to only 40% after NRP ($p = 0.0053$). Following a reduction of DWIT (15 min), DCD liver recipients achieved comparable survivals with NRP (80%).

Interpretation High-risk DCD livers benefit more from HOPE-treatment, either immediately in the donor or after cold storage. Comparative prospective clinical studies are required to translate the results.

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Keywords: Normothermic regional perfusion; Hypothermic oxygenated perfusion; Mitochondria; Liver transplantation; Donation after circulatory death; Ischemia reperfusion injury

Research in context

Evidence before this study

Dynamic liver preservation techniques are increasingly explored in clinical practice with impact on posttransplant results and costs. While the oxygen supply is the common feature of most approaches, the debate on the best perfusion temperature is ongoing.

Added value of this study

This is an initial comparison of normothermic regional perfusion (NRP) and hypothermic oxygenated perfusion (HOPE) in a comprehensive, experimental model of donation after circulatory death (DCD) liver transplantation.
Greater ischemia-reperfusion injury (IRI), triggered by more mitochondrial injury was observed during NRP and particularly with increasing asystolic donor warm ischemia time (DWIT). In contrast, HOPE-treatment, applied either in the donor, e.g., before standard cold storage, or afterwards, as currently done clinically, showed better mitochondrial protection with less IRI-associated inflammation. A direct consequence was the better recipient survival with both HOPE techniques. Of note, posttransplant survival rates with NRP were improved to levels comparable with HOPE after a reduction of donor injury (e.g., asystolic DWIT) to values, similar to what is observed in the human setting in different European countries, where NRP is applied successfully.

Implications of all the available evidence

While an early reoxygenation before cold storage, as done with NRP, is beneficial compared to cold storage alone, the results point to a better mitochondrial protection when oxygen is re-introduced under hypothermic conditions first, particularly when the donor injury is elevated. The results of this study warrant a confirmation in a prospective clinical trial.

Introduction

Liver transplantation from donation after circulatory death (DCD) donors is routinely performed in many countries with an increasing use of dynamic preservation technologies. For example, in situ machine perfusion of the donor liver, called normothermic regional perfusion (NRP), is performed immediately after circulatory death. This procedure represents currently the standard preservation technique for DCD livers and kidneys in France, Spain and parts of the United Kingdom (UK).[1–3] In contrast, other countries, including Switzerland and the Netherlands, apply preferably an end-ischemic hypothermic oxygenated perfusion (HOPE) in the recipient centre, after initial super rapid organ procurement and liver transport on ice.[4,5] Combining both approaches may even be more beneficial, as done in Italy, where donor warm ischemia times (DWIT) are prolonged due to the mandatory 20 min stand-off period, required by law prior to circulatory death declaration.[3] DCD organs are therefore routinely procured in Italy with NRP, followed by static cold storage (CS), plus an additional end-ischemic HOPE in the vast majority of transplantations.[6] This concept has achieved excellent outcomes, comparable to DBD liver transplants, and with low rates of primary non-function (PNF) and ischemic cholangiopathy (IC), despite the use of DCD livers with extended criteria.[3,7] Equally, a recent randomized controlled trial (RCT) demonstrated the protective effect of end-ischemic HOPE, without initial NRP, on the frequency and severity of clinically relevant ICs after DCD liver transplantation.[4]

Despite the clinical benefit of both procedures, NRP and HOPE, a mechanistic comparison in standardized models has never been performed. The aim of this study was therefore, to explore ischemia reperfusion injury in a highly standardized experimental transplant model.

Methods

All materials and methods are presented according to the ARRIVE guidelines and further detailed in the Supplementary Document.

Study design and experimental groups

A rodent model of normothermic regional perfusion (NRP) was developed, initially with healthy livers to confirm adequate perfusion of the abdominal organs (DBD + NRP group). Next, donor rats were attributed to 30 min or 60 min asystolic donor warm ischemia time (DWIT), which started at the point of cardiac arrest. At the end of DWIT, livers were either directly procured and exposed to static cold storage (DCD + CS group; without additional machine perfusion), or underwent in-situ NRP (DCD + NRP) or HOPE in the donor (donor_HOPE). Healthy DBD livers without relevant cold storage or machine perfusion served as controls. In a first step, livers were evaluated during and at the end of preservation, corresponding to the situation directly before transplantation. Male Brown Norway rats were

randomly allocated to the following experimental groups (Fig. 1):

1) Healthy Livers exposed 2 h NRP (n = 8, each, DBD + NRP group)
2) Livers exposed to DWIT with subsequent 2 h NRP, followed by procurement and 5 h standard cold storage (n = 8, each, DCD + NRP group)
3) Livers exposed to DWIT with direct cold flush, procurement and 5 h standard cold storage (n = 8, each, DCD + cold storage group)
4) Livers exposed to DWIT with in situ HOPE for 2 h and 5 h standard cold storage (n = 8, each, DCD + donor_HOPE group)
5) Livers exposed to DWIT with subsequent cold flush and 5 h cold storage and 2 h ex-situ HOPE (n = 8 each, DCD + HOPE_Rec group)

In a second step, the set of experiments was repeated with the same experimental groups with 30 min DWIT and additional liver transplantations. Recipient animals and livers were evaluated during the first week after implantation. A more detailed description of the experimental groups is provided in the supplementary material.

Model of normothermic regional perfusion (NRP)

The rodent model of in-situ abdominal NRP was established carefully considering parameters currently seen in clinical practice. In healthy controls (DBD + NRP group) and DCD liver donors allocated to NRP (DCD + NRP), the abdominal aorta and inferior vena cava (IVC) were both prepared and cannulated distal to the renal veins (Fig. 1, Figure S1). The thoracic aorta and cava vein were both clamped to achieve the solitary perfusion of the abdominal compartment. The 2 h NRP was started when the vessels were cannulated (DBD-NRP) or at the end of DWIT (DCD + NRP groups). One additional rat served as a blood donor. The conditions during NRP were maintained according to the clinical experience in countries with regular use of this preservation technique. At the end of NRP, livers were sampled and assessed or procured after a flush with 5–6 mL precooled, heparinized UW solution for additional cold storage and transplantation (Fig. 1). More details can be found in Supplementary Materials.

Model of in-situ hypothermic oxygenated perfusion (donor_HOPE)

The same perfusion circuit was used to perform in-situ, abdominal hypothermic oxygenated perfusion (HOPE;

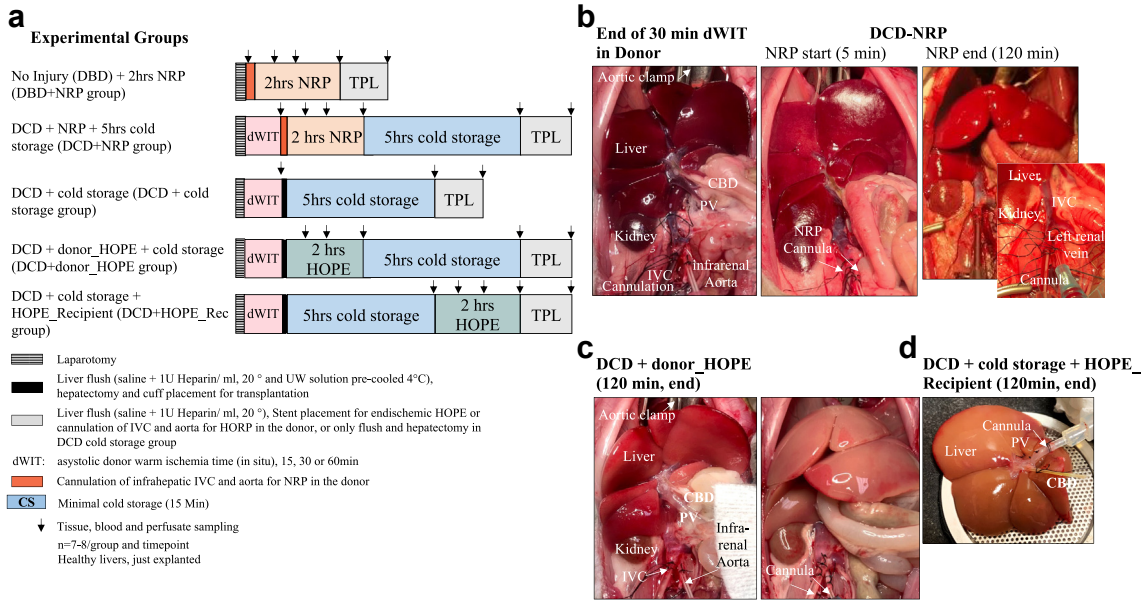


Fig. 1: **Experimental design**. The different experimental groups (a) and the preservation concepts are shown, including normothermic regional perfusion (NRP) (b) and hypothermic oxygenated perfusion (HOPE) in the donor, i.e., before static cold storage (DCD + donor_HOPE; c) and after static cold storage (DCD + HOPE_Rec; d). Livers are well and homogenously perfused with both techniques at the end of 120min of NRP (b), HOPE in the donor (donor_HOPE, c) and HOPE in the Recipient (HOPE_Rec, d). Main anatomical structures are shown including infrahepatic vana cava (IVC) and infrarenal aorta, liver and right kidney. The cannula placed for abdominal NRP are shown in the infrarenal aorta and IVC (b and c). The livers portal vein was cannulated as per standard for the ex-situ HOPE (HOPE_Recipient; d) (DBD: donation after brain death; DCD: donation after circulatory death; DWIT: donor warm ischemia time (asystolic); NRP: normothermic regional perfusion; CS: static cold storage; Tpl: transplantation.

DCD + donor_HOPE). The HOPE technique is routinely applied ex-situ after cold storage on a perfusion device.[8,9] Following abdominal vessel cannulation as described above, donor blood was flushed out and discarded. Using a switching valve the circuit was turned into the recirculation mode for donor_HOPE treatment. The perfusion was pressure controlled with a limit of maximal perfusion pressure of 3 mmHg. The perfusate was highly oxygenated (pO_2: 60–80 kPa) and the temperature was adjusted to 8–10 °C (Fig. 1). At the end of 2 h donor HOPE, livers were excised and assessed or underwent 5 h standard cold storage for later assessment or transplantation.

Ex-situ (end-ischemic) hypothermic oxygenated perfusion (HOPE-Recipient)

To compare donor HOPE treatment before cold storage with the current clinical "standard", in a separate experimental group, rodent DCD livers were flushed, procured and cold stored for 5 h before portal vein stenting and ex-situ HOPE-treatment as described previously.[9–11] Ex-situ HOPE was performed for 2 h at 8–10 °C with a maximal perfusion pressure of 3 mmHg. Similarly, as during the donor_HOPE, perfusates were highly oxygenated (Fig. 1).

Orthotopic liver transplantation

Before liver transplantation (e.g., directly after procurement in DBD controls, or after cold storage in DCD + NRP or DCD + donor_HOPE groups, or after cold storage + additional HOPE_Recipient), livers underwent an additional flush with precooled UW with subsequent non-arterialized liver transplantation, according to the technique by Kamada et al. (Figure S2).[8–10,12]

Endpoints and analyses

Liver tissues, perfusates and donor plasma were obtained at different timepoints (Fig. 1). The perfusion quality was assessed through perfusate pH, oxygen partial pressures and electrolytes from perfusate obtained during NRP. The levels of liver injury and inflammation were explored during NRP, donor_HOPE and HOPE_Recipient; in the next set of experiments after additional cold storage (e.g., before implantation). Parameters included perfusate Lactate, Aspartate-aminotransferase (AST), Alanine-aminotransferase (ALT). Reactive oxygen species (ROS)-induced downstream inflammation was further explored through quantification of 8-hydroxy-2-deoxy Guanosine (8-OHdG) and high mobility group box protein-1 release (HMGB-1) by ELISA in perfusate and recipient plasma. The downstream activation of parenchymal and non-parenchymal cells was further detected by perfusate levels of Toll-Like-Receptor-4 (TLR-4), TLR-9, NLRP-3 and Intercellular-Adhesion-Molecule-1 (ICAM-1) by ELISA in perfusate and recipient plasma. This perfusate analysis was paralleled by Quantitative Real-Time Polymerase Chain Reaction (PCR) analysis on obtained liver tissues.

Additionally, mitochondria were isolated from liver tissues to assess Adenosine-trisphosphate (ATP)-content together with precursors using targeted liquid chromatography-mass spectrometry (LC-MS) as previously described.[13] ATP was also measured from liver tissues as previously described.[14,15] Flavin-mononucleotide (FMN) was measured in perfusate and tissue using LC-MS and fluoroscopy. The membrane–bound complex I FMN content was also measured in isolated mitochondria after separation of respiratory chain complexes by high resolution Clear Native gel.[16] The percentage of structurally modified complex I was assessed through the quantification of the NDUFS-1 subunit in perfusates. To parallel the assessment of mitochondrial metabolism and inflammation, various staining procedures were performed, including complex I (NDUFS-1), TUNEL, Haematoxylin-Eosin (H&E), TLR-4 and NF-kB.

Ethics

Completeness, plausibility, and validity of the data were independently verified (by R.P., M.FC., L.M., J.E., D.M., A.G., P.D, and A.S.). The experiments were approved by the Italian Ministry of Health (authorization 90/2020-PR) and carried out according to European Union (EU) directive guidelines (2010/63/EU) and Italian legislation (DLgs 26/2014) for animal care procedures at "Centro di Stabulazione degli animali da laboratorio, Ce.S.A.L" of Florence University.

Statistics

Data are presented as median and interquartile range (IQR) or n and %. Statistical analysis was performed using the non-parametric Mann-Whitney-Wilcoxon U-test to compensate for the samples size. Multiple group comparisons were done with one-way Anova and regression analysis was used to determine correlations between specific groups. Correlations were calculated using non parametric tests (Spearman r test). The * included in the figures highlight significant differences between the different groups presented (*$p < 0.05$, **$p < 0.01$, *** <0.001; ****$p < 0.0001$). GraphPad Prism, version 7.0, San Diego, CA, USA was used for the analysis.

Role of funders

The funding sources (Swiss National Science Foundation, University Careggi, OTT, the Max Planck Society and the NIH) had no role in study design, data collection, data analysis, interpretation or writing of the manuscript.

Results

Normothermic regional perfusion of healthy controls and DCD livers

Based on current clinical concepts and previous studies with large animals, a new rodent model of NRP was developed (Fig. 1a and b, Figure S1).[17–19] First, this NRP-model was applied for a period of 2 h in abdominal compartments from healthy DBD donors (DBD + NRP, healthy control), simulating optimal conditions. In these experiments, perfusion parameters, haemoglobin and electrolytes were all kept in physiological ranges and livers were well oxygenated.[20,21] Correspondingly, perfusate liver enzymes, lactate, DAMP and cytokine levels remained low throughout 2 h of NRP in healthy DBD donors, further confirmed by minimal activation of non-parenchymal liver cells (Fig. 2a–f).

Second, NRP was applied in DCD donors after 30 and 60 min of asystolic DWIT (DCD + NRP). Perfusion conditions in the DCD model were identical compared to DBD livers, with no differences in NRP blood flow, oxygenation and electrolyte (Fig. 2b). After 30 min in-situ asystolic DWIT, we found however an increase of liver transaminases and lactate in the circulating blood during NRP (Fig. 2c), corresponding to increased DAMP-signalling, toll-like-receptor-4 (TLR-4) activation and histological signs of liver injury (Fig. 2d–f). All of these parameters increased further significantly with extension of asystolic DWIT to 60 min (Fig. 2c–f). The clinically relevant parameter lactate was gradually cleared during NRP, comparable to human DCD donors, with however high levels beyond 10 mg/dl in DCD livers with 60 min DWIT (Fig. 2c). An increasing donor risk was associated with significantly more signs of IRI-associated liver injury and inflammation during NRP.

In-situ HOPE (donor HOPE) and DCD livers

Third, we applied in-situ HOPE (DCD + donor_HOPE) instead of NRP after complete flush out of donor blood and switch to an oxygenated hypothermic perfusate, using the same NRP-circuit (Figs. 1 and 3a). In contrast to NRP, where oxygen was provided under normothermic conditions, we observed significantly lower IRI-signs and inflammation during in-situ HOPE, despite increasing asystolic DWIT (Fig. 3b–e). The entire spectrum of parameters obtained from perfusates and liver tissues, including liver transaminases, 8-OHdG, Hmgb-1 and cytokine-levels remained low, despite increasing DWIT to 60 min. Liver tissues demonstrated only minimal signs of injury and TLR-4-positivity, a surrogate of non-parenchymal cell activation (Fig. 3b–e).

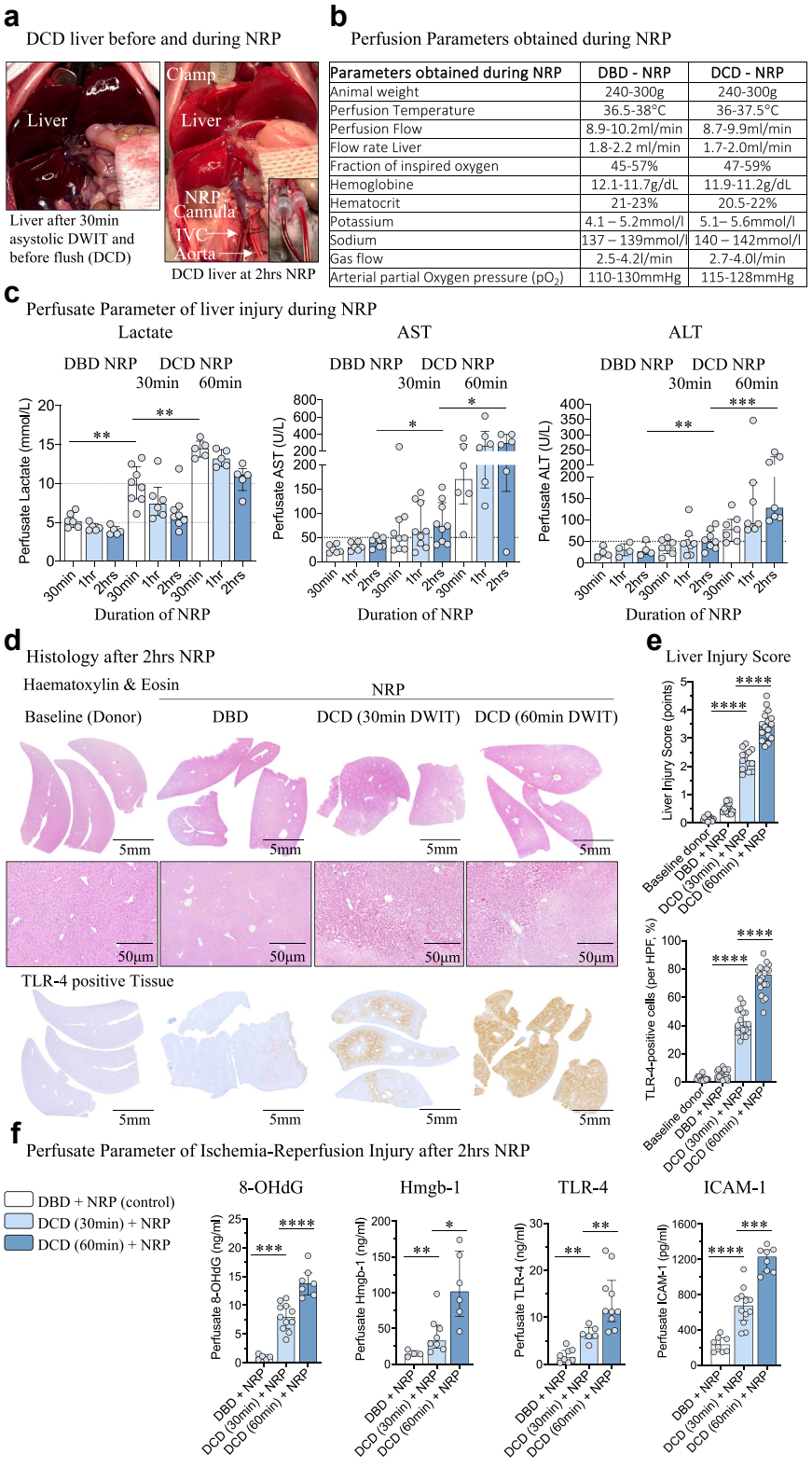
Effect of cold ischemia after NRP or HOPE

In a fourth step, we added a period of additional 5 h cold storage to NRP-treated, or in-situ HOPE-treated DCD livers with 30 min asystolic DWIT. This was done to simulate organ transport and evaluate the situation immediately before transplantation. Liver tissues were obtained immediately before transplantation to assess mitochondrial injury and tissue inflammation (Figs. 4a and 5a). While NRP resulted in higher tissue ATP levels compared to untreated cold-stored DCD livers, we found a significantly reduced mitochondrial complex I activity and more complex I flavin mononucleotide (FMN)–loss after NRP (Fig. 4a–c). Complex I activity and function were preserved best through HOPE (in-situ before additional cold storage or end-ischemic after additional cold storage, Fig. 4a–d), together with highest ATP levels after HOPE (Fig. 4a–d). This finding was further supported by a clear correlation between mitochondrial complex I activity, mitochondrial complex I FMN-content, and loss of FMN into NRP perfusates (Fig. 4b–g). Structural complex I changes during IRI were previously described in the literature. Liver tissues were therefore assessed for NDUFS-1 content before liver transplantation in all groups with 30 min DWIT. Along with complex I protection and higher activity, lower NDUFS-1 positivity and perfusate levels were seen after in-situ HOPE or end-ischemic HOPE compared to NRP (Fig. 4e and g).

Further downstream such mitochondrial injury was linked to IRI-associated cell activation as shown by a higher tissue positivity for TLR-4 and NFκB (Fig. 5b and c). In contrast, both HOPE techniques, in-situ in the donor and end-ischemic after cold storage led to less tissue inflammation as demonstrated by lower TLR-4 and NFκB-tissue positivity (Fig. 4d and e). These results with the level of inflammation in the tissue before liver implantation were further paralleled by perfusate analyses for IRI-associated markers at the end of NRP, donor HOPE and end-ischemic HOPE (Figure S3). Mitochondrial complex I activity, injury and FMN content were further linked to the level of TUNEL positivity before transplantation (Figure S4).

Effect of NRP, in-situ HOPE and end-ischemic HOPE on posttransplant outcomes

In a last step, we performed orthotopic liver transplantation with NRP, in-situ HOPE- and end-ischemic HOPE-treated DCD livers (30min asystolic DWIT). Healthy (DBD + NRP) and cold stored DCD livers served as controls. While all recipients of NRP-treated DBD livers (healthy controls) achieved an excellent survival, additional injury conveyed through 30 min asystolic DWIT translated into significantly lower survival rates after NRP (40%), similar to cold stored control DCD livers. In contrast, DCD liver recipients after donor or recipient HOPE were found with better outcomes and 88.9% and 85.7% survival rates, respectively. Based on the clinical literature with expertise from French and Spanish colleagues who describe excellent results after NRP provided the DWIT is not prolonged, we decided to reduce the a-systolic DWIT in an additional NRP group to 15 min. After a reduction of DWIT to 15 min, DCD liver recipients had better survival



Parameters obtained during NRP	DBD - NRP	DCD - NRP
Animal weight	240-300g	240-300g
Perfusion Temperature	36.5-38°C	36-37.5°C
Perfusion Flow	8.9-10.2ml/min	8.7-9.9ml/min
Flow rate Liver	1.8-2.2 ml/min	1.7-2.0ml/min
Fraction of inspired oxygen	45-57%	47-59%
Hemoglobine	12.1-11.7g/dL	11.9-11.2g/dL
Hematocrit	21-23%	20.5-22%
Potassium	4.1 – 5.2mmol/l	5.1– 5.6mmol/l
Sodium	137 – 139mmol/l	140 – 142mmol/l
Gas flow	2.5-4.2l/min	2.7-4.0l/min
Arterial partial Oxygen pressure (pO_2)	110-130mmHg	115-128mmHg

Fig. 2: **Liver ischemia-reperfusion-injury and inflammation during NRP.** Rodent DCD livers before and **during NRP (a), perfusion parameters obtained during NRP of abdominal compartments of DBD and DCD donors (b), parameters measured from obtained perfusate**

(80%) with NRP (Fig. 6a and b). Such clinical findings were further supported by higher recipient plasma liver enzymes, FMN, more TUNEL positivity, higher Hmgb-1 and proinflammatory cytokine levels in the NRP (30 min DWIT) group. The reduction of asystolic DWIT to 15min in the NRP group led also to lower inflammation and less NFkB-positivity in liver tissues after transplantation (Fig. 6c–e & Figure S5) (Fig. 7).

Discussion

This experimental study provides an experimental comparison of two main dynamic preservation methods, currently used to improve and assess human DCD livers before transplantation. The following main findings were present: First, no relevant ischemia-reperfusion-injury (IRI) was seen during NRP of healthy DBD livers or DCD grafts with short asystolic donor warm ischemia time (DWIT, 15 min). Secondly, with increasing donor injury, e.g., prolonged DWIT, significantly more signs of IRI were seen with NRP, corresponding to the known IRI-cascade after transplantation. Third, this was in clear contrast to an application of HOPE after cold storage, or with application of HOPE already in the donor. Fourth, such results were even more pronounced after transplantation. This study parallels previous analyses, where mechanisms of mitochondrial protection through cold oxygenation were described.[13]

Despite the different perfusion modalities, all concepts of dynamic organ preservation follow the main principle to reintroduce oxygen into ischemic tissue, either under warm conditions (e.g., initially during NRP or instead of cold storage with normothermic machine perfusion (NMP)) or hypothermic (e.g., after cold storage in the recipient centre) with the HOPE-concept.[4,5,22] Both preservation techniques, NRP in the donor and HOPE after cold storage are increasingly used for DCD livers in clinical practice. Controlled DCD livers are procured and evaluated with NRP in France and Italy (100% of cases) and in Spain (68% of cases).[1,23,3,24] Despite the overall good results, a few differences reported from such countries deserve a more detailed discussion, supporting the findings of the present study. Based on different regulations and parameter definitions in combination with centre experience, the cumulative donor risk appears different among well-known centres. Recent studies describe the lowest donor risk in Spain with a reported median total DWIT (time from withdrawal to circulatory donor death) between 16 and 23.7 min in recent series.[1,25–27] Such results parallel our experimental findings and the lower inflammation during NRP after reduction of the asystolic DWIT to 15 min. In contrast, and also based on the obligatory 20 min donor stand-off period, DCD livers are transplanted with a median of 50 min total DWIT in Italy.[3,28] The majority of DCD livers undergoes additional HOPE-treatment after cold storage with subsequent excellent outcomes and no relevant graft loss due to IC.[3,6] Similar differences are found with the duration of NRP. While the current practice in France and Italy is 3–4 h, in Spain the median NRP duration is shorter with 101.5 min to 121 min.[1,25–27] With the aim to achieve a model closely related to clinical practice, we opted for an NRP duration of 2 h. A recent study from France demonstrated no differences in posttransplant outcomes with an NRP duration between 2 and 4 h.[29]

Our results underline, that exposure to oxygen after significant ischemia can be dangerous and leads to activation of inflammatory cascades. This process can obviously be downscaled, when lowering the temperature during reperfusion of hypoxic tissues, i.e., with the application of HOPE, in contrast to oxygenated reperfusion at normothermic conditions. Our results parallel recent clinical findings with NRP in different countries. For example, authors from Italy recently tried to reduce the number of DAMPs and cytokine molecules released during NRP by adding a cytosorb filter to the NRP-circuit.[30] A French study demonstrated inferior outcomes after transplantation of DCD livers with NRP-procurement when the accepted donor liver had prolonged functional donor warm or cold ischemia times of >30min or >8 h, respectively.[31] The majority of DCD grafts beyond acceptance criteria was lost (n = 16/21) either initially due to poor function or later with liver cancer recurrence or the development of ischemic cholangiopathy, despite the use of NRP.[31] A group from Spain with a large NRP experience has recently analysed national data to identify predictors of inferior outcomes. Authors recommend to combine NRP with ex-situ machine perfusion when the overall risk is too high, e.g., when the cold ischemia time is prolonged with >7 h and in technically complex recipient, including cases with retransplantation.[32] The first combined cases were recently performed in Spain. Pueyo-Périz et al. describe

samples during NRP, including Lactate and liver transaminases (AST and ALT) (c), n = 5–11 per group and timepoint. Representative histological images of rodent livers at the end of 2 h NRP, H&E and TLR-4 staining (d), quantification of histological images obtained at the end of 2 h NRP calculating the Suzuki score and the percentage of TLR-4-positive cells per specimen and area assessed, blinded for experimental group, n > 12–15 HPF (e), perfusate parameters of IRI quantified at the end of 2 h NRP including 8-OHdG, Hmgb-1, TLR-4 and ICAM-1 (n = 6–12 per group and timepoint) (f), data presented as median and IQR; Non-parametric Mann–Whitney test: *p < 0.05; **p < 0.01; ****p < 0.0001. ALT: Alanine-Aminotransferase; AST: Aspartate-Aminotransferase; DBD: donation after brain death; DCD: donation after circulatory death; DWIT: donor warm ischemia time; Hmgb-1: high mobility group box protetin-1; ICAM-1: Intercellular adhesion molecule-1; IRI: ischemia-reperfusion-injury; IVC: infra-hepatic vena cava; NRP: normothermic regional perfusion; TLR-4: Toll-like-receptor 4; 8-OHdG: 8-hydroxy-2-deoxy Guanosine.

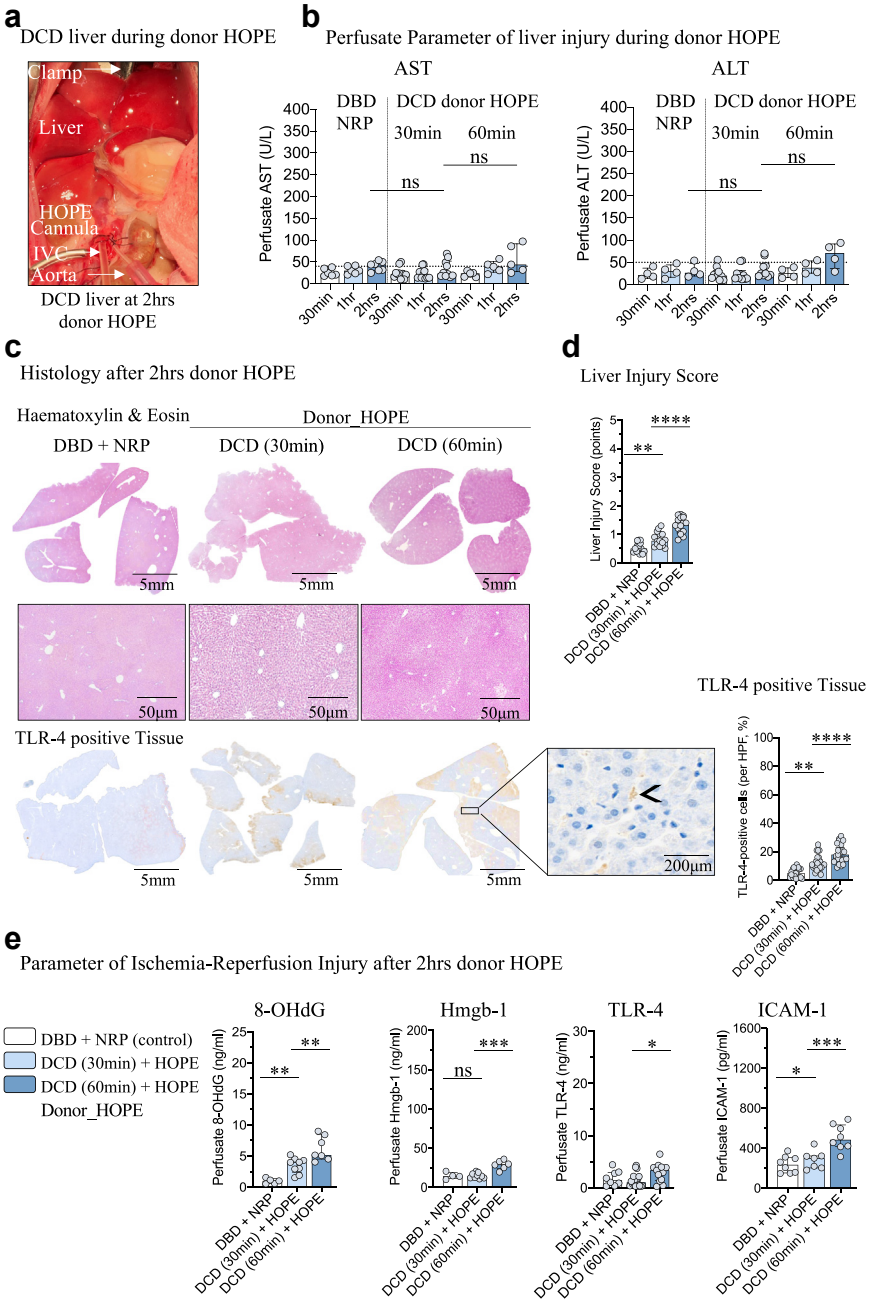


***Fig. 3:* Liver ischemia-reperfusion-injury and inflammation during donor HOPE**. Rodent DCD livers during donor HOPE (a), perfusate parameters of liver IRI during donor HOPE (n = 5–10 per group and timepoint) (b), representative histological images of rodent livers at the end of 2 h donor HOPE, H&E and TLR-4 staining (c), quantification of histological images obtained at the end of 2 h NRP calculating the Suzuki score and the percentage of TLR-4-positive cells per specimen and area assessed (n > 12–15 HPF) (d), perfusate parameters of IRI quantified at the end of 2 h donor HOPE including 8-OHdG, Hmgb-1, TLR-4 and ICAM-1 (e). Data presented as median and IQR; Non-parametric Mann–Whitney test: *p < 0.05; **p < 0.01; ****p < 0.0001. ALT: Alanine-Aminotransferase; AST: Aspartate-Aminotransferase; DBD: donation after brain death; DCD: donation after circulatory death; DWIT: donor warm ischemia time; Hmgb-1: high mobility group box protetin-1; HOPE: hypothermic oxygenated perfusion; ICAM-1: Intercellular adhesion molecule-1; IRI: ischemia-reperfusion-injury; IVC: infra-hepatic vena cava; NRP: normothermic regional perfusion; TLR-4: Toll-like-receptor 4; 8-OHdG: 8-hydroxy-2-deoxy Guanosine.

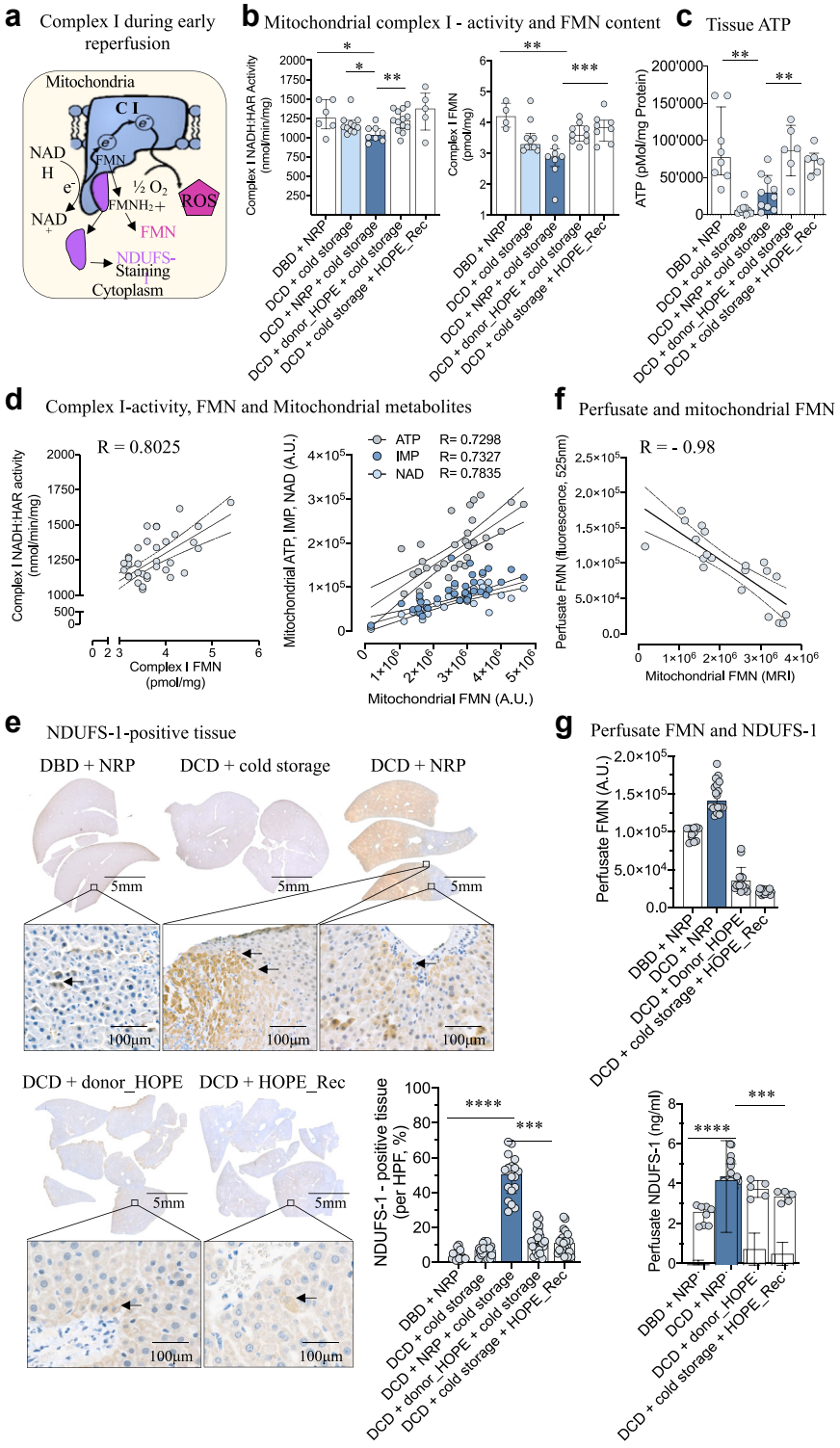


***Fig. 4:* Mitochondrial injury with different preservation strategies before transplantation**. Complex-I during early reperfusion (a), isolated mitochondria and liver tissues were analysed at the end of preservation and before transplantation; Complex-I activity and FMN content and were measured through complex I WB (b); liver tissue ATP content was measured (c). Complex-I activity and metabolites correlated well with mitochondrial FMN content as measured through MS (d). Based on structural changes in complex I at reintroduction of oxygen into ischemic tissue, NDUFS-1 is released from complex I into cellular plasma (where it can be stained (e) (n > 12–21 HPF)); Perfusate FMN inversely correlates

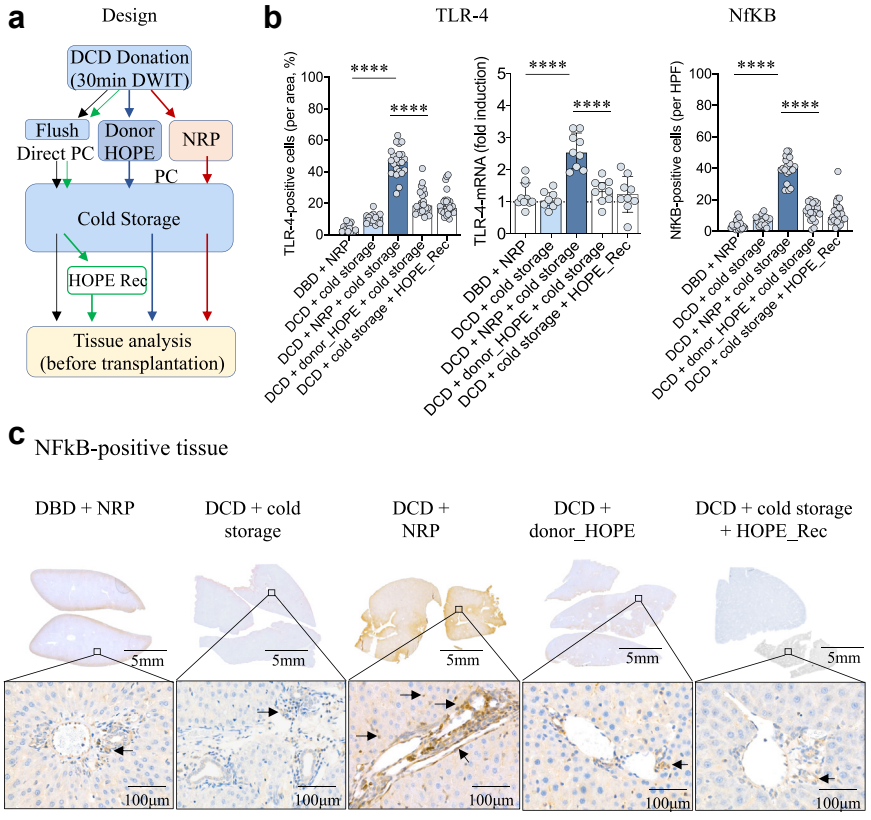


***Fig. 5*: Comparison of various preservation strategies before transplantation**. Overview on experimental groups and timepoint when liver tissues were obtained for analysis (a), tissue expression of TLR-4 and NFkB analysed through real-time PCR (b, n = 8–9 per group) and histology (c, n > 11–23 HPF), before liver implantation (2 h NRP or donor HOPE with cold storage, at the end of recipient HOPE), data presented as median and IQR; Non-parametric Mann–Whitney test: *p < 0.05; **p < 0.01; ****p < 0.0001. DBD: donation after brain death; DCD: donation after circulatory death; DWIT: donor warm ischemia time; HOPE: hypothermic oxygenated perfusion; IRI: ischemia-reperfusion-injury; NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells; NRP: normothermic regional perfusion; TLR-4: Toll-like-receptor 4.

the resuscitation of controlled DCD livers with HOPE after previous NRP.[33]

In contrast, protective effects of HOPE compared to standard cold storage of DCD livers were recently demonstrated in a European RCTs with a significant reduction of the number and severity of IC,[4] confirming earlier preclinical studies.[9] Based on the general reduction of postreperfusion inflammation by HOPE, recipients in the perfusion group also experienced a 10% lower acute rejection rate [32]. The impact of endischemic HOPE on liver injury and recipient complications was also evident in three additional RCTs demonstrating lower early allograft dysfunction rates, less complications and reduced costs, with better graft survival and lower retransplantation rates.[34,35] The most recent multicentre RCT demonstrated the protection from liver related major complications and related graft loss.[36]

With the introduction of donor perfusion concepts in the 80ties and 90ties, techniques of donor perfusion were the research focus by many. Such concepts were however quite different from what is done clinically today and also from the here presented technique of NRP and donor HOPE. Oxygenated donor perfusion

with mitochondrial FMN assessed in all samples from groups where livers underwent perfusion (e.g., NRP, donor_HOPE and HOPE_Recipient) (f); NDUFS-1 was also measured in perfusates together with FMN, in all groups with perfusion (e.g., NRP, donor_HOPE and HOPE_Recipient) (g); data presented as median and IQR; Non-parametric Mann–Whitney test: *p < 0.05; **p < 0.01; ****p < 0.0001. ATP: adenosine trisphospate; DBD: donation after brain death; DCD: donation after circulatory death; DWIT: donor warm ischemia time; FMN: flavin-mononucleotide; HOPE: Hypothermic oxygenated perfusion; IMP: Inosine monophosphate; IRI: ischemia-reperfusion-injury; NAD: nicotine adenine dinucleotide, NDUFS-1: NADH:ubiquinone oxidoreductase 75 kDa Fe-S protein 1; NRP: normothermic regional perfusion; ROS: reactive oxygen species.

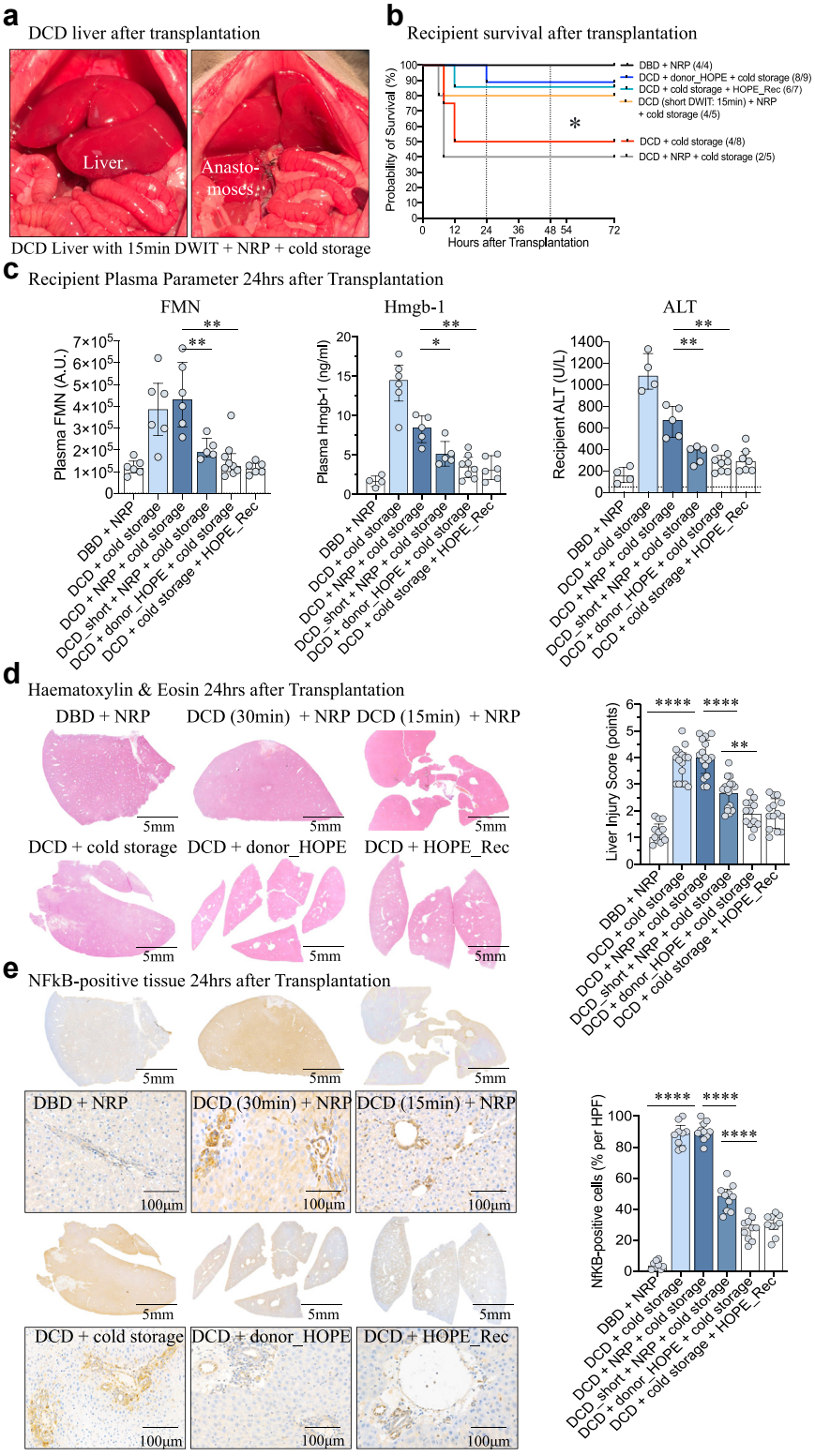


***Fig. 6:* Outcomes after transplantation of DCD livers with different preservation protocols**. DCD liver after transplantation (a), recipient survival within the first week after transplantation in all experimental groups (n = 4–9) (b), recipient plasma parameters of mitochondrial and liver injury within the first 24 h after transplantation, including FMN, Hmgb-1 and ALT (c, 5–9 per study group, plasma from 1 to 3 animals at

was extensively tested using cardiopulmonary bypass and ECMO technologies however initially mainly in uncontrolled donors with a focus on results after kidney transplantation showing relatively high rates of delayed graft and primary non functions.[37] Ko et al. performed eight transplantations with DCD kidneys procured with cold ECMO. Donors were perfused using an established ECMO-circuit at 4 °C with subsequent standard cold storage.[38] The main difference was the use of cooled donor blood. Farney et al. followed a slightly different approach and procured DCD kidneys and pancreas with cooling of the entire donor to 22 °C with subsequent ECMO with oxygenated donor blood. A total number of 53 DCD kidneys were transplanted with results comparable to DBD kidney transplantations.[39] While such early studies pushed the development of normothermic NRP circuits as they are used clinically today, no evidence for hypothermic donor perfusion with subsequent liver transplantation is currently available. And concepts of hypothermic organ perfusion have evolved significantly with the need for high perfusate oxygen levels,[22] however without the requirement of oxygen carriers, which may lead to higher levels of inflammation.[40] These findings were recently supported by a RCT assessing the impact of donor body cooling on outcomes after kidney transplantation.[41] Authors from the United States procured DCD kidneys from donors that underwent hypothermia at 35 °C. This concept of donor precooling was however inferior with higher rates of delayed graft function compared to end-ischemic ex-situ hypothermic kidney perfusion. This parallels previous experimental studies demonstrating that mitochondria work more efficient and are protected when oxygen is reintroduced at temperatures below the Arrhenius break point of 15 °C.[42] This study further supports previous evidence indicating that a proper cooling is required for an effective HOPE-treatment in both, the donor and also during an end-ischemic ex-situ approach in the recipient centre.

With this study we did not observe differences between HOPE in the donor or recipient centre, this might however change with different risk factors, such as longer cold storage. The application of HOPE in the donor may have additional benefits, as HOPE-treatment of an entire abdominal compartment might also protect other organs with a similar effect on mitochondria, previously described for the isolated end-ischemic hypothermic kidney, heart, lung and pancreas perfusion.[43–46] An initial cold oxygenation was recently found beneficial for DCD kidneys compared to the delayed introduction of oxygen after an episode of HMP with low oxygen levels or cold storage.[46] Such preclinical findings were paralleled by a European RCT showing a better initial kidney function and reduced rejection rates when HMP was performed with high partial pressures of oxygen.[47] Further studies are however required to determine the protective HOPE-effect in marginal livers and other solid organs with different cold storage durations before and after perfusion.

This study has strengths and limitations. First, although animal models have various advantages, assessing groups with a more standardized risk profile, our findings require confirmation in the clinical setting, ideally with an RCT comparing NRP and HOPE at the donor and the recipient site. Second, despite the higher tissue inflammation and NFkB-upregulation in portal triads after NRP, a marker predicting biliary injury, due to the short posttransplant follow-up no conclusion regarding the development of later biliary strictures can be made.

Third, further research is required to identify the acceptable duration of cold storage after NRP or donor HOPE, and also before end-ischemic HOPE in livers with different risk profiles. This may have significant impact on logistics with future use of dynamic preservation concepts in donor or organ treatment- and assessment-hubs with subsequent transport with cold storage after organ perfusion. Fourth, this study did not include a group with NRP and additional HOPE after cold storage in the recipient centre, representing the clinical model in Italy, which should be part of future analyses. Fifth, while the FMN-release from complex I during reperfusion is known since 1969 in different organs,[13,48–50] and is currently used as a viability marker during HOPE in some liver transplant centres, the real structural changes at the NADH-docking site of complex I during reperfusion are not well-understood yet. Finally, while the NRP-model was developed closely related to clinical practice, achieving physiological perfusion parameters comparable to healthy rodents, any perfusion model could be improved with a better technology and a more tailored perfusate composition in the future, which may lead to better recipient survival rates after NRP of high-risk

time of death was included), histological tissue assessment (n > 10–25 HPF) for H&E (Suzuki score) and NfkB-positivity 24 h after transplantation (d), c&d separate set of experiments with n = 4–9 transplantations per group; Following impaired survival after NRP with 30min asystolic DWIT, the injury was reduced to levels seen in clinical practice in European countries, the repetition of the experiments with 15min DWIT in the NRP group improved survival based on less IRI-associated inflammation (A to E); data presented as median and IQR; Non-parametric Mann–Whitney test: *p < 0.05; **p < 0.01; ****p < 0.0001, ALT: Alanine-Aminotransferase; DBD: donation after brain death; DCD: donation after circulatory death; DWIT: donor warm ischemia time; FMN: flavin-mononucleotide; Hmgb-1: high mobility group box protetin-1; HOPE: hypothermic oxygenated perfusion; IRI: ischemia-reperfusion-injury; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells; NRP: normothermic regional perfusion.

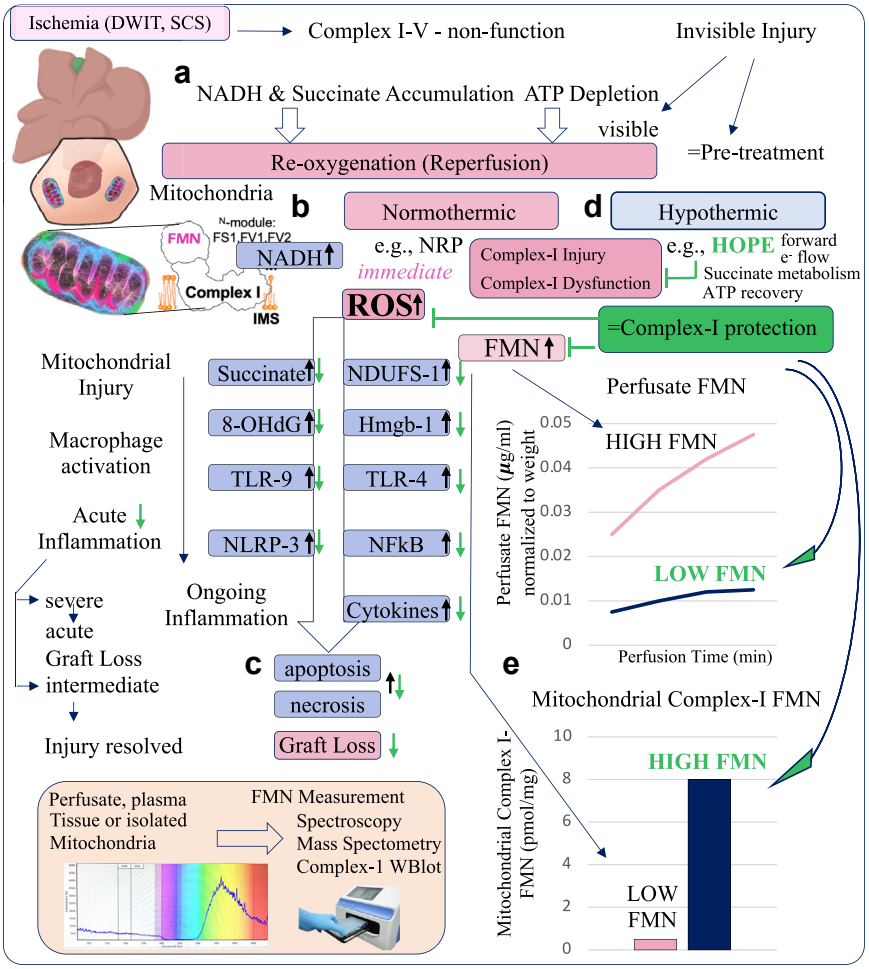


***Fig. 7*: Mechanism of Complex-I tiggered mitochondrial injury and downstream inflammation after ischemia-reperfusion**. As described by many, during ischemia the electron flow throughout the respiratory chain is on hold with subsequent energy depletion and accumulation of toxic metabolites, e.g., Succinate and NADH (a), with the aim to reduce the level of such molecules, they contribute to the retrograde electron flow (RET) when oxygen is reintroduced under normothermic conditions with complex I dysfunction and injury, depending on the previous donor quality or duration of ischemia (b), triggered by this initial complex I dysfunction, the IRI cascade is initiated with immediate ROS & FMN-release and continues when cells lyse and more molecules are release, including succinate, NDUFS-1, other DAMPs and cytokines with activation of other cells, which did not get hit severely yet. Severe acute and ongoing inflammation will result in graft loss. (c), The identification of strategies, which address this high complex I (-V) dysfunction during early reperfusion are of great importance, here described by the HOPE approach; in contrast to reoxygenation under normothermic conditions, this hypothermic perfusion technique reconditions mitochondrial respiration and metabolism protecting complexes I–V (d), together with immediate ROS, FMN is also released from complex I and triggers molecular changes in the NDUFS-1/V1-FMN position, leading to NDUFS-1 release and plasma positivity in the staining. High perfusate FMN correlates with low complex I FMN and vice versa (E). DCD: donation after circulatory death; DWIT: donor warm ischemia time; FMN: flavin-mononucleotide; Hmgb-1: high mobility group box protetin-1; HOPE: hypothermic oxygenated perfusion; IRI: ischemia-reperfusion-injury; NDUFS-1: NADH:ubiquinone oxidoreductase 75 kDa Fe-S protein 1; NF-kB: Nuclear factor kappa-light-chain-enhancer of activated B cells; NLRP-3: NLR family pyrin domain containing 3; NRP: normothermic regional perfusion; TLR-4: Toll-like-receptor 4; 8-OHdG: 8-hydroxy-2-deoxy Guanosine.

DCD livers. Further experimental studies and comparative RCTs are needed with NRP to identify and test modified perfusion conditions, including lower temperatures, various perfusate additives and to evaluate the effect of a pulsatile perfusion flow. Prolonged NRP perfusion might be required to overcome the initial IRI-hit and to develop more specific and mitochondria-focussed viability tests, as currently explored for ex-situ NMP.[51]

In summary, this study provides an experimental comparison on NRP and HOPE and the effect on mitochondrial and ischemia reperfusion injury. End-

ischemic or donor HOPE were both superior to NRP in a transplant model. These results should be further confirmed in a RCT.

Contributors

Conceptualization, R.P., P.M. and A.S.; Methodology, R.P., M.FC, F.A., L.M., D.M., A.G., P.D. and A.S.; Investigation, R.P., M.FC, N.N., J.E., L.M., L.B.B., R.X.S.S., M.P., D.D., D.M., A.G., P.D. and A.S.; Verification of data: R.P., D.M., A.G., A.S.; Writing original Draft, R.P. and A.S.; Writing Review & Editing, all authors; Funding Acquisition, P.M., M.F., A.P., F.M., P.D. and A.S., Resources, M.F., D.M., P.M., F.M., P.D., A.S.; Supervision, P.M., F.M.; All authors read and approved the final version of the revised manuscript.

Data sharing statement

Data supporting the figures and tables of this manuscript (analysis data) are available from the corresponding author upon reasonable request.

Declaration of interests

The authors have no conflicts of interest to disclose related to this study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104861.

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