Research Article

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Warm *vs.* cold perfusion techniques to rescue rodent liver grafts

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Background & Aims: A variety of liver perfusion techniques have been proposed to protect liver grafts prior to implantation. We compared hypothermic and normothermic oxygenated perfusion techniques in a rat liver transplant model, using higher risk grafts obtained after cardiac arrest (DCD).

Methods: Rat livers were subjected to 30 or 60 min *in situ* warm ischemia, without application of heparin. Livers were excised and stored for 4 h at 4 °C, mimicking DCD organ procurement, followed by conventional organ transport. In experimental groups, DCD liver grafts received a 4 h normothermic oxygenated perfusion through the portal vein and the hepatic artery instead of cold storage. The perfusate consisted of either full blood or leukocyte-depleted blood (normothermic groups). Other livers underwent hypothermic oxygenated perfusion (HOPE) for 1 h after warm ischemia and 4 h cold storage (HOPE group). Liver injury was assessed during machine perfusion and after isolated liver reperfusion, and by orthotopic liver transplantation (OLT).

Results: DCD livers, subjected to normothermic perfusion, disclosed reduced injury and improved survival compared to cold storage after limited warm ischemia of 30 min (70%; 7/10), but failed to protect from lethal injury in grafts exposed to 60 min warm ischemia (0%; 0/10). This finding was consistent with Kupffer and endothelial cell activation in cold stored and normothermic perfused livers. In contrast, HOPE protected from hepatocyte and non-parenchymal cell injury and led to 90% (9/10) and 63% (5/8) animal survival after 30 and 60 min of donor warm ischemia, respectively.

Conclusions: This is the first evidence that HOPE is superior to normothermic oxygenated perfusion in a clinically relevant model through modulation of the innate immunity and endothelial cell activation.

Introduction

Several competing liver perfusion concepts have been developed over the past decade to improve or rescue injured liver grafts prior to implantation. While successes have been shown in different animal models [1–9], the human application of these liver perfusion techniques remains limited [10–12]. The proposed clinically relevant strategies include: (a) a continuous normothermic oxygenated perfusion with full blood, applied immediately and continuously after procurement [13], (b) a similar perfusion technique, however, using diluted isolated red blood cells [13,14], and (c) a hypothermic oxygenated perfusion (HOPE) without blood cells, applied after cold storage for only one hour prior to graft implantation [3,7,15,16]. No conclusive comparison of these approaches is currently available.

Our aim was, therefore, to compare clinically relevant perfusion techniques, using an established DCD liver transplant model. As pre-treatment of potential donors is prohibited in many countries due to ethical reasons, we adapted our study to the human situation of Maastricht III donors, i.e. in controlled cardiac death, omitting any interventions in donors before organ procurement [17]. With these prerequisites, we investigated four different strategies using normothermic perfusion with full blood and with leukocyte-depleted blood, cold storage combined with HOPE, and conventional static cold storage as a control. The end points included a variety of markers of injury during machine perfusion, isolated liver reperfusion, and eventually animal survival after orthotopic liver transplantation (OLT).

Materials and methods

Animals

Male Brown Norway rats (250–320 g) were used in all experiments. Animals were maintained on standard laboratory diet and water according to the Swiss Animal Health Care law. All experiments were approved by the animal ethics committee. Anaesthesia during liver procurement and transplantation was maintained with isoflurane.

Keywords: DCD liver; Hypothermic oxygenated perfusion; Normothermic oxygenated liver perfusion; Liver transplantation; Survival.

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Abbreviations: DCD, donation after cardiac death; HOPE, hypothermic oxygenated perfusion; UW solution, University of Wisconsin solution; HMGB-1, high mobility group box-1 protein; 8-OHdG, 8-hydroxy-2-deoxy guanosine; H&E, haematoxylin and eosin; ROS, reactive oxygen species; DAMPs, damage associated molecular pattern signalling; CD68, macrosialin; ELISA, enzyme-linked immunosorbent assay; SECs, sinusoidal endothelial cells; WI, warm ischemia; IPRL, isolated perfused rat liver; TLR-4, Toll-like receptor 4; MPO, myeloperoxidase; IL-6, interleukin 6; IL-10, interleukin 10; IL-1β, interleukin 1β; TNF-α, tumor necrosis factor alpha; ICAM-1, intercellular adhesion molecule-1; NSE, neuron specific enolase; CXCL-5 (ENA-78), epithelial neutrophil-activating protein 78.

Study design

We opted for an established rodent DCD model [3,16,18] with induction of cardiac arrest by hypoxia due to incision of the diaphragm without prior heparinization or portal clamping. The period of *in situ* warm ischemia started from the point of cardiac arrest.

In a first step we compared the extent of graft injury in healthy livers (non – DCD, no injury) and DCD livers during either conventional cold storage or during treatment by two types of machine perfusion. We selected two donor warm ischemia periods, i.e. 30 and 60 min asystolic warm ischemia, reflecting either extended DCD livers or non-viable DCD livers, as determined in pilot transplant experiments. In a second step, we analysed reperfusion injury on the isolated liver perfusion model and also graft injury and survival after liver transplantation of cold stored or machine perfused livers.

For this purpose we chose the following experimental groups (Supplementary Fig. 1):

(1) Healthy livers (non-DCD) without warm ischemia, exposed to minimal cold storage (15 min UW solution) and subsequent 4 h normothermic oxygenated perfusion with full rat blood served as a proof of concept (normothermic control group, no injury + normotherm).
(2) DCD livers exposed to 30 min or 60 min of *in situ* warm ischemia followed by 4 h normothermic oxygenated machine perfusion with full rat blood (DCD + normothermic full blood group).
(3) DCD livers exposed to 30 min of *in situ* warm ischemia followed by 4 h normothermic oxygenated machine perfusion with isolated erythrocytes (DCD + normothermic leukocyte-depleted blood group).
(4) DCD livers exposed to 30 min or 60 min of *in situ* warm ischemia followed by 4 h cold storage (UW solution) (DCD + cold storage).
(5) DCD livers exposed to 30 min or 60 min of *in situ* warm ischemia and 4 h of cold storage followed by 1 h of hypothermic oxygenated perfusion (DCD + cold storage + HOPE group).

After a downtime, i.e. warm ischemic injury of 30 or 60 min, livers were flushed *in situ* with 6 ml heparinized (1 U/ml) saline at room temperature and UW solution at 4 °C via the portal vein only. Livers were then excised (weight 10.14 ± 2.73 g) and placed in precooled UW solution (4 °C). From this stage, livers were allocated to the normothermic perfusion groups (2a: DCD + normothermic full blood, 2b: DCD + normothermic leukocyte-depleted blood), to the untreated cold storage group (3: DCD + cold storage) or to the HOPE group (4: DCD + cold storage + HOPE). In the HOPE group, livers received stents for the portal vein, the supra-hepatic and the infra-hepatic vena cava. An additional cannula was inserted in the hepatic artery in the normothermic groups. All perfused liver grafts were placed to a physiologic position in an organ bath for most optimal flow and temperature (Supplementary Fig. 1).

Normothermic oxygenated machine liver perfusion

Normothermic liver perfusion was performed with either oxygenated diluted full blood [2] or with a leukocyte and platelet depleted blood perfusate, as the presence of blood cells potentially multiplies reperfusion injury [14]. In case of full blood perfusion, we used 30 ml autologous rat blood, diluted with 20 ml Krebs Henseleit Buffer (haematocrit 14-16, white blood cell count 2000/mm^3). The perfusate was substituted with nutrients (Nutriflex® 1 ml/h), taurocholic acid (20 mg), bicarbonate 8.4% (1 ml), prostacyclin (1 µg/h) and amoxicillin (500 mg) according to Brockmann *et al.* [2].

For normothermic perfusion experiments with leukocyte depleted blood as perfusate, we isolated erythrocytes out of full rat blood and added afterwards isolated red blood cells to Williams E medium, according to Op den Dries *et al.* [14] (haematocrit 14-16, leukocyte count <100/mm^3).

Livers were perfused in both normothermic groups continuously at 37 °C (open bath thermostat, Huber, Germany) through the portal vein at 8 mmHg by pressure control (gravity), and in pulsatile manner through the hepatic artery (flow controlled, 6 ml/min, roller pump: Masterflex, Cole-Parmer Instrument Company, USA). Perfusates were actively oxygenated (pO_2 40–50 kPa; hollow fiber oxygenator D150, Hugo Sachs Elektronik).

HOPE treatment

DCD livers to be cold perfused (3: DCD + CS + HOPE group) were connected after 4 h cold storage to the precooled perfusion device and perfused for 1 h through the portal vein with a constant perfusion pressure of ⩽3 mmHg, and active oxygenation (pO_2 50–60 kPa; hollow fiber oxygenator D150 Hugo Sachs Elektronik). We used as perfusate 50 ml recirculating modified starch-free UW solution according to previous experiments [3,15,16,19]. Perfusion box and perfusate were maintained at 4 °C by an open bath thermostat (Huber, Germany).

Ex-vivo reperfusion on the isolated perfusion rat liver model (IPRL)

In all experiments livers were flushed with 5 ml cold UW solution after machine perfusion or cold storage. Livers were afterwards connected to the isolated perfused rat liver (IPRL) device. Prior to reperfusion, livers were left untouched in the chamber for 15 min to simulate a rewarming period during vascular anastomoses in OLT. Afterwards, livers were reperfused by pressure control (8 mmHg) through the portal vein for 3 h with a new oxygenated (pO_2 50–60 kPa) perfusate including 50 ml of diluted heparinized autologous blood (haematocrit 14-16), gentamicin (60 mg/kg), and taurocholic acid (20 mg/50 ml). Perfusion box and perfusate were maintained at 37 °C by an open bath thermostat (Huber, Germany).

Orthotopic liver transplantation

Before liver transplantation, livers of all experimental groups were disconnected from the perfusion device and placed in a precooled petri dish. All livers were flushed with 5 ml cold UW solution and cuffs were inserted in the portal vein and the infra-hepatic vena cava (8 ± 1.8 min). Subsequently, non-arterialized OLT was performed according to the technique by Kamada *et al.* [20].

End points

Liver graft injury was assessed during machine perfusion (n = 10, each group), during *ex-vivo* reperfusion (n = 10, each group) and after transplantation (n ⩾10 each group).

Hepatocyte injury was measured by AST release in perfusate and animal plasma (multiple biochemical analyzer DRI-CHEM4000i; FUJIFILM; Japan). Liver function was determined by bile production during machine perfusion or *ex-vivo* reperfusion.

Mitochondrial function was analysed by liver tissue ATP as previously described [16,21]. Oxidative damage of DNA by reactive oxygen species was detected using an 8-hydroxy-2-deoxy guanosine (8-OHdG) Elisa (Abnova, KA0444). Nuclear subcellular injury was measured by release of the high mobility group box protein-1 (HMGB-1, IBL, SHINO Test, ST51011) using a specific ELISA (IBL International GmbH). Activation of non-parenchymal cells and cytokine release was detected by a Toll-like receptor 4 ELISA (TLR-4; MyBiosource, MBS705488), tumor necrosis factor alpha (TNF-α) ELISA (R&D; RTA00), interleukin-6 (IL-6) ELISA (R&D; R6000B), interleukin-1β (IL-1β; R&D; RLB00) ELISA, and interleukin-10 (IL-10; R&D; R1000) ELISA. Neutrophil activation was assessed in the perfusate during and after machine preservation and in animal plasma after transplantation, using the following specific ELISAs: Epithelial neutrophil-activating protein 78 (ENA 78; MyBiosource; MBS 817719) and the neurone specific enolase (NSE; MyBiosource, MBS292549).

Quantitative real-time polymerase chain reaction

Total RNA was extracted from liver tissue using Trizol reagent (Invitrogen, Basel, Switzerland). After generation of complementary DNA (ThermoScript reverse-transcription polymerase chain reaction system; Invitrogen), quantitative real-time polymerase chain reaction amplification and data analysis were performed on the ABI Prism 7000 sequence detector system (PE Applied Biosystems, Rotkreuz, Switzerland). TaqMan gene expression assays (PE Applied Biosystems) for IL-6 (Rn01410330_m1), Hmgb-1 (Rn02377062_m1), TNF-α (Rn99999017_m1), IL-10 (Rn00563409_m1), IL-1β (Rn00580432_m1), TLR-4 (Rn00569848_m1), CXCL-5 (Rn00573587_g1), NSE (Rn00595017_m1), ICAM-1 (Rn00564227_m1), were used and normalized to 18S rRNA (control reagents; PE Applied Biosystems).

Histology and immunohistochemistry

The following staining procedures were performed after machine perfusion, before transplantation, after *ex-vivo* reperfusion, and after OLT (12 h, 1 week): hematoxylin-eosin (H&E) staining for necrosis, Toll-like-receptor-4 (TLR-4; LS-B2070), CD68 (macrosialin; MCA341R) and myeloperoxidase (MPO; RB-373-A1) stainings for neutrophils, von-Willebrand factor staining (vWF; A0082) and intercellular-adhesion-molecule-1 staining (ICAM-1; sc-1511-R) for endothelial