CHAPTER

Organ-specific responses during brain death: increased aerobic metabolism in the liver and anaerobic metabolism with decreased perfusion in the kidneys

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ABSTRACT

Introduction

Metabolic assessment of brain-dead donors is a potentially novel strategy to assess and target graft quality prior to transplantation. This study investigated metabolic changes, tissue perfusion, oxygen consumption, and mitochondrial function, in the liver and kidneys following brain death (BD).

Materials and methods

BD was induced in mechanically-ventilated rats by inflation of an epidurally-placed Fogarty catheter; sham-operated rats served as controls. A 9.4T preclinical MRI system measured hourly oxygen availability (BOLD-related R2*) and perfusion (T1-weighted). After 4 h, hepatic and renal tissue was collected, mitochondria isolated and assessed with high-resolution respirometry. Quantitative proteomics, qPCR, and biochemistry was performed on stored tissue/plasma.

Results

Following BD, the liver increased glycolytic gene expression (Pfk-1) with decreased glycogen stores, while the kidneys increased fermentation-related expression (LdhA). During BD, oxygen consumption significantly increased in the liver, while tissue perfusion decreased in the kidneys. Mitochondrial respiration and complex I/ATP synthase activity were unaffected in both organs following BD.

Discussion

In conclusion, the liver responds to increased metabolic demands during BD, enhancing aerobic metabolism with functional mitochondria. In contrast, the kidneys shut down metabolically, shifting towards anaerobic energy production while renal perfusion is decreased. Our findings highlight the need for an organ-specific approach to assess and optimise graft quality prior to transplantation.

INTRODUCTION

The shortage of donor organs suitable for transplantation remains a major healthcare problem. Despite various strategies to expand the donor pool, such as the increased use of marginal, non-heart beating, or living donor grafts, most organs transplanted worldwide are still obtained from brain-dead donors. However, compared to living donor transplantation, transplantation of brain-dead organ grafts leads to higher rejection rates and inferior long-term outcomes. Thus, the current challenge is to use all available organs including the suboptimal and concurrently improve transplantation outcomes.

Brain death (BD) causes complex disturbances in body homeostasis. BD is the result of increased intracranial pressure (ICP), which leads to progressive ischemia of the cerebrum, brain stem, and spinal cord. Consequently, these changes trigger a sympathetic response with catecholamine release, which in turn causes systemic vasoconstriction and decreased flow through peripheral organs including the liver and kidneys. Furthermore, impairment of the hypothalamus and pituitary gland results in hormonal disturbances, including reduced levels of circulating triiodothyronine, vasopressin, and cortisol. Eventually, ischemia of the spinal cord results in the loss of sympathetic tone in the peripheral vascular bed, potentially impacting future organ grafts.

These systemic disturbances may have detrimental effects on future donor organs. In the liver and kidneys, this has been evidenced by increased injury biomarkers in plasma and apoptosis, immune activation, inflammation, and oxidative stress in tissue. Treatments administered to brain-dead donors to improve post-transplantation graft, and thus recipient, survival may be of benefit. However, systematic reviews show no consistent evidence for the effectiveness of any such treatments. Hence, new strategies are needed to assess and optimise organ quality prior to transplantation by the use of innovative organ-specific treatments given either to the donor or during ex vivo machine perfusion.

In an attempt to identify alternatives to assess or improve donor-organ quality, we initiated this study to investigate alterations during BD in the liver and kidneys. Under normal circumstances, both organs are metabolically active, i.e. the liver fulfils a synthesis and detoxification function and the kidneys an excretory one. The liver is considered the predominant site for carbohydrate, lipid, and amino acid metabolism. Nevertheless, the kidneys are responsible for supplying up to half of the total blood glucose levels during prolonged fasting or starvation. These metabolic processes are normally tightly regulated as perturbances in metabolic checkpoints e.g. changes in nutrient or oxygen supply can initiate both apoptosis and necrosis. Glucose levels in particular are under tight neuro-endocrine control through actions of insulin, catecholamines, thyroid hormone, and cortisol. Considering the dysregulation of this neuro-endocrine system, as well as tissue injury and apoptosis during BD, it is conceivable that metabolism changes during BD and that these changes reflect or even influence the quality of transplantable organs.

Few studies have explored metabolic changes in the brain-dead donor. Novitzky et al. reported decreased metabolite utilisation and accumulation of fatty acids and lactate in plasma, suggesting a shift from aerobic to anaerobic metabolism. Similar results in the myocardium of brain-dead pigs pointed towards increased anaerobic metabolism in combination with decreased ATP levels. These results suggest that the observed metabolic changes are caused either by impaired oxygen utilisation due to a primary...
metabolic (i.e. mitochondrial) impairment or alternatively, by impaired oxygen delivery due to changes in tissue perfusion. Mitochondrial impairment can cause increased anaerobic ATP production as well as oxidative stress and has previously been observed in the hearts of brain-dead pigs and the muscle fibres of brain-dead, human subjects. However, mitochondrial function following BD in the liver and kidneys has not been examined. Alternatively, the observed anaerobic alterations could result from changes in peripheral perfusion in the hemodynamically unstable brain-dead donor. Animal studies have shown decreased perfusion of the liver and kidney immediately following the Cushing response as well as traumatic brain injury. However, perfusion of the liver and kidneys during BD has not yet been explored.

The purpose of this study was to investigate the influence of BD on systemic and specifically hepatic and renal metabolism, in a rodent BD model. We hypothesised that previously observed anaerobic changes during BD originated at least in part from either mitochondrial dysfunction or impaired peripheral perfusion in the liver and kidneys. To test this hypothesis we used repetitive in vivo magnetic resonance imaging (MRI) throughout BD to visualise tissue perfusion and oxygenation. Furthermore, mitochondrial function was assessed by measuring in vitro mitochondrial respiration as well as mitochondrial proteomics. Our findings showed different metabolic responses in the liver versus kidneys during BD, suggesting the need for an organ-specific approach to assess and optimise graft quality prior to transplantation.

METHODS

Brain death model

Sixteen male, adult Fisher F344 (Harlan, UK) rats (250-300 g) were randomly assigned to the BD (n = 8) or sham-operated (sham) group (n = 8). The experimental protocol was approved by the Danish Animal Experimentation Inspectorate, under The Ministry of Food, Agriculture, and Fisheries (Approval no. 2014-15-2934-01007). All animals received care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. Rats were kept in cages in a 12:12 h light-dark cycle, with a temperature of 21°C ± 2°C, and humidity levels of 55% ± 5%. Animals had ad libitum access to a standard rodent diet (Altromin, Lage, Germany) and tap water.

The brain death model used was previously described by Kolkert et al., with the following adaptations to ensure MRI compatibility. Animals were anesthetized using sevoflurane with 100% O2, intubated via a tracheostomy, and ventilated (MR-compatible Small Animal Ventilator, SA Instrument, Inc. NY, USA) with the following ventilation parameters: tidal volume of 7 ml/kg of body weight (kg) per stroke, positive end expiratory pressure of 3 cm H2O with an initial respiratory rate of 120 per min, and corrected based on end-tidal CO2 (ETCO2). Continuous MAP monitoring and volume replacement was performed via cannulas that were inserted in the femoral artery and vein, respectively. A frontotemporal hole, drilled for the epidural placement of a no. 4 Fogarty catheter used to induce BD (Edwards Lifesciences Co, Irvine, CA), a second hole was drilled contralaterally for ICP monitoring with a 24G cannula. BD was induced by inflation of the Fogarty catheter; an initial inflation of the MAP rose above 80 mmHg. BD was confirmed when the ICP superseded the MAP. Possible reasons for exclusion of animals from the study was the inability to confirm BD or maintain a normotensive MAP. Rocuronium (0.1 mg/ml at 0.3 ml/h) was administered to avoid movements during MR scanning. Sham animals underwent an identical surgical procedure, without insertion of the Fogarty catheter, while anaesthesia lasted the entire 4 h of experimental time.

After 4 hrs, the experiment was terminated as previously described and the liver, kidneys, plasma and urine were harvested. Tissue from the liver and one kidney were used for mitochondrial isolation. Additional tissue, plasma and urine were stored.

Plasma injury markers, blood gas analysis, and metabolites

Plasma levels of AST, ALT, creatinine, urea, LDH, glucose, and lactate were determined at the clinical chemistry laboratory of the University Medical Centre Groningen according to standard procedures. Results from one sham animal resulted in supraphysiological values of plasma markers, which was confirmed statistically with an outlier test. As a result, this animal was removed from the analyses. Blood gas analyses were performed immediately after aortic puncturing using ABL725 analysers (Radiometer Medical Aps, Brønshøj, Copenhagen, Denmark) to determine the pH, partial pressure of oxygen, partial pressure of carbon-dioxide, haemoglobin and SaO2. Samples containing blood clots were excluded from analyses.

Acylcarnitine analysis was performed using a method previously described. Supernatant acylcarnitine concentrations were measured with an API 3000 LC-MS/MS, equipped with a Turbo ion spray source (Applied Biosystems/MDS Sciex, Ontario, Canada).

Glucose metabolism and glycogen storage

Periodic acid–Schiff staining

To determine glycogen tissue concentrations, a PAS staining was performed in paraffin embedded tissue samples. Next, all slices were scanned and 10 pictures per slice at 20 x of magnification used to estimate the positive PAS area with ImageJ software. The final value per slice was recorded as the mean of positive areas.

RNA isolation, cDNA synthesis, and Real-Time quantitative PCR

From whole liver and kidney sections, we isolated total RNA using TRIzol (Life Technologies, Gaithersburg, MD), with a method previously described. From whole liver and kidney sections, we isolated total RNA using TRIzol. From whole liver and kidney sections, we isolated total RNA using TRIzol.

| Table 1. Primer sequences used for Real-Time PCR. |
|-----------------------------------------------|-----------------------------------------------|
| **Gene** | **Primers** | **Amplicon size (bp)** |
| PKC-1 | 5'-CTGCTCTCAACGATCGTACGCT-3' | 91 |
| PK | 5'-TGCGATCAAGGACGAACT-3' | 81 |
| LDHA | 5'-ATGGAGGCGGCGTCATG-3' | 88 |
| PCK | 5'-CTGGAAGGGAATGCTCTC-3' | 74 |
| HO-1 | 5'-CTGCGATACCACTCTGGAGAT-3' | 74 |
Mitochondrial respiration

Mitochondrial isolation
After 4 h of BD, organs were removed and placed into ice-cold 0.9% KCl solution. A differential centrifugation procedure was used to isolate either mitochondria from 1.5 g of liver tissue or a whole kidney. The total volume of working reagent required was determined by calculation of the protein concentration in the mitochondrial suspensions with a BCA protein assay kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA).

High resolution respirometry
The rates of oxygen consumption in isolated mitochondria (0.4 mg/ml of mitochondrial protein) were measured at 37 °C using a two-channel high-resolution Oroboros oxygraph-2k (Oroboros, Innsbruck, Austria). Assay medium contained: EGTA (0.5 mM), MgCl2 (3 mM), KH2PO4 (10 mM), Lactobionic acid (60 mM), Taurine (20 mM), HEPES (20 mM), D-Sucrose (110 mM), and bovine serum albumin (BSA, 1 mg/ml, pH 7.2). The substrates for oxidation were: (i) pyruvate (5 mM) + malate (2 mM), (ii) succinate (5 mM) + rotenone (1 μM), (iii) glutamate (5 mM) + malate (5 mM), and (iv) palmitoyl-CoA (25 μM) + L-carnitine (2 mM) + malate (2 mM). To reach maximal ADP-stimulated oxygen consumption (state 3) hexokinase (4.8 U/ml), glucose (12.5 mM), and ADP (1 mM) were added. Resting state oxygen consumption rate (state 4) was measured after we blocked ADP phosphorylation with carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 2 μM). The oxygen consumption rate in the uncoupled state (state U) was determined after addition of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 2 μM). The respiratory control ratio (RCR) was calculated by dividing oxygen consumption rate in state 3 by that of state 4. Data acquisition (4 Hz sampling frequency) and analysis were performed using DatLab software version 5 (Oroboros, Innsbruck, Austria).

Magnetic resonance imaging (MRI) assessment of oxygen consumption (BOLD) and perfusion (ASL)
Animals were placed in a MRI compatible animal-bed (Rapid Biomedical, Würzburg, Germany) and the following parameters controlled: rectal temperature, blood and intracranial pressure, ETCO2, and pulse oximetry. MRI data was collected with an Agilent 9.4 T preclinical MRI system (Agilent Technologies, Yarnton, UK), containing a 72-mm quadrature 1H transmit/receive volume coil (Rapid Biomedical, Würzburg, Germany).

A high-resolution coronal spin-echo sequence (Agilent Technologies, Yarnton, UK), containing a 72-mm quadrature 1H transmit/receive volume coil (Rapid Biomedical, Würzburg, Germany).

BOLD MRI relies on differences in oxygenated and deoxygenated haemoglobin concentrations in blood vessels and surrounding tissue. These differences cause contrast in MRI influencing the so-called spin-spin relaxation rate (R2*), allowing R2* values to be correlated to oxygen consumption (when external factors such as regional perfusion are accounted for) and inversely correlated to oxygen availability of a specific region of interest (i.e. low R2* values indicate low oxygen consumption yet high oxygenation)29,30. R2* can be calculated using an oxygenation-dependent sequence (T2*-weighted) and obtained using an axial 1H-multi-echo gradient-echo sequence, covering the entire abdomen with 32 slices using the following sequence parameters: matrix of 128×128, FOV of 80×80 mm2, flip angle of 90°, TR of 800 ms, TE of 2, 4, 6, 8, 10, 12, 14, and 16 ms, number of transients of 2, and 2 mm thickness. R2* values calculated at baseline (time 0 h) served as an internal control to calculate relative changes in oxygenation, a measure superior to absolute values.

ASL MRI relies on a difference in the T1-weighted signal of inflowing blood compared to that of the tissue of interest, allowing estimation of relative changes in tissue perfusion. For T1-measurements a single-slice segmented Look-Locker sequence with a gradient-echo readout was used to acquire T1-weighted data. The following sequence parameters were used: matrix of 128×128, FOV of 80×80 mm2, flip angle of 8°, TR of 3 ms, TE of 2 ms, inversion times (TI) of 150, 250, 400, 600, 900, 1200, 2500, 4000 ms, and 2 mm thickness.

Vmnl (Agilent Technologies) was used for image reconstruction and volumetric analysis. Manually drawn regions of interest were encompassed around the liver and kidneys. Quantitative T2* maps were calculated from pixel-by-pixel analysis using a nonlinear least-squares fit to the logarithmic magnitude vs. TE. Quantitative T1 maps were was calculated from a three-parameter fit applied to the inversion recovery Look-Locker sequence, using the mathematical approach given by Ramasawmy et al30.

Mitochondrial proteomics, complex I and ATP synthase activity, and tissue ATP levels
Targeted, quantitative mitochondrial proteomics
In isolated mitochondria, we quantified a selection of mitochondrial proteins involved in the substrate transport, FAO and TCA cycle, using isotopically-labelled standards (1C-labeled lysines and arginines). These were derived from synthetic protein concatamers (QiComCAT) (PolyQuant GmbH, Bad Abbach, Germany), using a method previously described31.

Hepatic and renal ATP concentrations
Frozen liver and kidney tissue was cut into 20 mm slices; 650 mg of these slices were used to determine ATP content according to standard procedures32.

Complex I and ATP synthase enzyme activity measurements
Mitochondria were isolated as previously described33, then diluted in PBS, lysed by sonication, and centrifuged at 600 g for 10 min at 4 °C. Protein concentration was determined in the supernatant using a BCA protein assay kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA).

Activity of complex I was monitored spectrophotometrically at 600 nm and 37 °C, as previously described34. Rotenone-sensitive complex I activity was calculated using the molar extinction coefficient of DCIP, equal to 21000 M−1 cm−1 and expressed as nmol/min/mg protein35. The activity of ATP synthase was measured spectrophotometrically at 340 nm and 37 °C, as previously described36. Oligomycin-sensitive ATP synthase activity was calculated using the molar extinction coefficient of NADH, equal to 6220 M−1 cm−1 and expressed as nmol/min/mg mitochondrial protein36.

Oxidative stress markers
Determination of oxidative damage through quantification of lipid peroxidation
Lipid peroxidation product MDA was quantified in liver and kidney homogenates (20 μl) by measuring the formation of thiobarbituric acid reactive substances with a method previously described37.

Gene expression of protective protein Heme oxygenase-1
Gene expression of Hoo-1 was determined with Real-time PCR as described previously with the primer set outlined in Table 1.
Statistical Analyses

For *R2 BOLD and T1 data, we estimated that we would need a total of 8 animals per group to detect a clinically significant difference with an α of 0.05, power of 80%, using a two-tailed test. Descriptive statistics were done to confirm that the data met the assumption of equal distributions of residuals. A linear mixed model was used with repeated measures over time to analyse the impact of the treatment (BD or sham) on BOLD and ASL in the liver and kidney, with fixed effects of time, treatment group, and the interaction of treatment and time (IBM SPSS Statistics 23). This model was chosen because it takes the dependency of the measurements across time into consideration, and prevented list-wise deletion caused by missing data points. The model selection for covariance parameters was chosen based on the best fit according to the Bayesian Information Criterion. When comparing two independent groups at a single time point, the non-parametric Mann-Whitney test was used to identify significant differences between the groups (n = 8 in each group), with Prism 6.0 (GraphPad Software Inc, CA, USA). To confirm abnormal results, a boxplot was performed to identify extreme outliers and considered significant when they scored > 3 x IQR compared to the other values with IBM SPSS Statistics 23. All statistical tests were 2-tailed and p < 0.05 was regarded as significant. Results are presented as mean ± SD (standard deviation).

RESULTS

Brain death parameters

As an internal control for the BD model, declaration of BD was confirmed when the ICP superseded the mean arterial pressure (MAP) and consequently cerebral perfusion pressure (CPP) was lower than 0 mmHg (Fig S1). Induction of BD showed a uniform MAP pattern consistent with previous studies\(^\text{1-17}\), with a mean time 29.3 ± 6.0 min to declare BD declaration. MAP of all animals was maintained above 80 mmHg throughout the experiment without the use of vasopressors or colloids. One out of eight experimental brain-dead animals had a CCP higher than 0 mmHg due to an obstruction of the ICP catheter, but as it showed a characteristic MAP profile and absent corneal and pupillary reflexes, the animal was included in the study (Fig S1).

Plasma functional and injury markers, metabolites, and pH following brain death

In plasma of brain-dead animals, increased levels of hepatic injury marker aspartate transaminase (AST, p = 0.001) but not alanine transaminase (ALT, p = 0.829) were found compared to sham controls (Fig 1A-B). Renal functional markers urea (p = 0.006) and creatinine (p = 0.001) were also increased following BD (Fig 1C-D). Lactate dehydrogenase (LDH) levels tended to be higher in the brain-dead group (p = 0.053, Fig 1E). Plasma glucose concentrations were significantly reduced following BD (p = 0.005, Fig 1F), yet lactate levels (p = 0.450, Fig 1G) and pH (p = 0.073, Fig 1H) were not different between the two groups. Results from one sham animal indicated supraphysiologic values of these plasma markers, which was confirmed statistically with an outlier test. As a result, this animal was removed from the analyses.

Acylcarnitine analysis showed that the concentrations of short-chain acylcarnitines (C3, C4, and C5: the degradation products of branched-chain amino acids) were significantly increased in the plasma of brain-dead animals (C2 and C5: p < 0.05, and C4: p < 0.01, Fig 2). Measurements of mitochondrial fatty acid β-oxidation (FAO) metabolites in plasma showed slightly elevated long-chain (C14 – C18) acylcarnitines concentrations, yet minimal changes in medium-chain (C6 – C12) acylcarnitine concentrations (C6, C12:1, and C14: p < 0.05, C18: p < 0.05, Fig 2).

Glucose metabolism and glycogen storage in the liver and kidneys following brain death

Expression of the glycolytic enzyme phosphofructokinase-1 (Pfk-1) was increased in the liver (p = 0.001), but not the kidney (p = 0.319) of brain-dead versus sham animals (Fig 3A). mRNA levels of glycolytic enzyme pyruvate kinase (Pk) did not differ between groups the liver (p = 0.336) and kidney (p = 0.130) (Fig 3B). The gluconeogenic enzyme pyruvate carboxylase (Pc) was similarly expressed in both groups in the liver (p = 0.093) and kidney (p = 0.293), whereas PEP carboxykinase-1 (Pck-1) expression was significantly lower in the kidneys of brain-dead compared to sham animals (p < 0.001) yet not different between groups in the liver (p = 0.694, Fig 3C). Expression of the fermentation-related enzyme lactate dehydrogenase A (Ldha) was not different between groups in the liver (p = 0.190), whereas increased expression was observed in the kidney of brain-dead versus sham groups (p = 0.038) (Fig 3D). Liver glycogen levels estimated with Periodic Acid-Schiff (PAS) staining showed a decrease in positively stained areas in the liver (p = 0.026), but not the kidney (p = 0.151) of brain-dead versus sham animals (Fig 3F).

No changes in mitochondrial respiration in the liver and kidneys following brain death

Mitochondrial function was assessed by measuring O2 consumption rates in isolated hepati- and renal mitochondria when oxidising four different substrate combinations in different metabolic states (Fig 4A-D). There were no significant changes in the maximal ADP-stimu- lated O2 consumption rate (state 3) in both the liver and kidney, indicating that the capac- ity to produce ATP through the oxidative phosphorylation pathway was not affected by
BD. Mitochondrial quality control was assessed with the respiratory control ratio (RCR), as a means to detect any changes in oxidative phosphorylation capacity related to tightness of mitochondrial coupling. The RCRs were not significantly different in brain-dead compared to sham animals when tested with any of the four substrate combinations (Fig 4E-H).

Figure 2. Brain death increased fatty acid β-oxidation. After 4 h of experimental time, plasma concentrations of saturated and unsaturated acylcarnitines were measured in sham and brain-dead rats with different carbon (C) chain lengths: short (C3 – C5), medium (C6 – C12), and long (C14 – C18) chain acylcarnitines. Data are represented as mean ± SD, n = 8 per group (* p < 0.05, ** p < 0.01, compared to sham).

Figure 3. Carbohydrate metabolism-related gene expression profiles and glycogen content in the liver and kidney after 4 h of brain death. Relative gene expression of glycogen related genes A1 Phosphofructokinase-1 (Pfk-1) and B Pyruvate Kinase (Pk), C fermentation related gene Lactate dehydrogenase A (Ldha), and gluconeogenesis related genes D Pyruvate carboxylase (Pc) and E PEP carboxykinase 1 (Pck-1). F) Periodic acid–Schiff staining of glycogen, overall quantification at 20x magnification. Results are presented as mean ± SD, n = 8 per group (* p < 0.05, ** p < 0.01).

Increased hepatic oxygen consumption (BOLD) and decreased renal perfusion (ASL) following brain death

Blood oxygen level dependent (BOLD) MRI relies on differences in oxygenated and deoxygenated haemoglobin concentrations in blood vessels and surrounding tissue, which in turn causes contrast in the spin-spin relaxation rate (R2*), allowing this rate to be correlated to oxygen availability. R2* baseline values were not different between sham and brain-dead animals in the liver and kidneys (Fig 5A, C, E). Using a linear mixed model for R2* BOLD values in the liver, we found a significant interaction between time and treatment group (p < 0.001), showing significant changes between groups over time (Fig 5A). Estimated effects in the liver were: BOLD BD = 0.157 + 0.009 * time; BOLD sham = 0.168 – 0.011 * time. In the kidneys, there was no significant effect of treatment and time nor a significant interaction effect between groups (Fig 5C, E).

Arterial spin labelling (ASL) MRI relies on a difference in the T1-weighted signal of inflowing blood compared to that of the tissue of interest. This signal difference can be used to estimate relative changes in tissue perfusion. In the liver, there was no significant effect of treatment and time nor a significant interaction effect between groups (Fig 5B). In contrast, in each of the kidneys, the linear mixed model for relative T1-weighted perfusion found significant interactions between time and treatment group (p < 0.05) in both kidneys (Fig 5D, F). This indicates that there are significant changes between the two treatment groups over time. Estimated effects for the left kidney were: ASL BD = 0.429 – 1.356 * time; ASL sham = -0.638 + 1.193 * time. Estimated effects for the right kidney were: ASL BD = -0.624 – 0.305 * time; ASL sham = -0.524 + 0.305 * time.
Increased metabolism-related protein expression in the liver and decreased expression in the kidney

Using a targeted, quantitative proteomics approach, we quantified 50 proteins involved in oxidative phosphorylation, tricarboxylic acid cycle (TCA), FAO, and substrate transport, as well as several antioxidant enzymes, in isolated hepatic and renal mitochondria. In the liver of brain-dead animals, we observed increased protein concentrations of peptides involved in substrate transport (Ucp2), the connection between glycolysis and TCA cycle (Dld and Dlat), and FAO (Acadm and Acadvl) \( (p < 0.05, \text{Fig 6}) \). Interestingly, most significant changes in the kidney showed decreased concentrations. These proteins were related to complex I (Ndufs1), TCA cycle (Aco2, Fh, and Suclg2) and the connection between FAO and electron transport chain (Etfdh), and FAO (Hadhb) \( (p < 0.05, \text{Fig 6}) \). The expression of two renal proteins, involved in substrate transport (Ucp2) and the TCA cycle (Dlat), was significantly higher in brain-dead compared to sham animals \( (p < 0.05, \text{Fig 6}) \).

To estimate how these changes in protein expression could influence the metabolic status of the potential grafts, cellular ATP content was measured. We showed reduced ATP levels in both the liver and kidney of brain-dead animals \( (p < 0.001, \text{Fig 5A}) \), suggesting a decreased bio-energetic status in both organs following BD.

The significantly different renal expression of a complex I peptide, as well as lower ATP levels in both liver and kidney, led us to investigate the activities of complex I and ATP synthase individually. We observed no differences in complex I and ATP synthase activity comparing brain-dead versus sham animals (Fig 5B, C).

Hepatic and renal oxidative stress markers

Tissue oxidative stress markers were assessed to evaluate how the observed changes in perfusion and oxygenation affected the liver and kidneys. Malondialdehyde (MDA), which relates to the amount of lipid peroxidation by reactive oxygen species (ROS), was increased in the kidney \( (p = 0.003) \), but not the liver \( (p = 0.442) \), of brain-dead compared to sham animals (Fig S3A). Furthermore, expression of the stress-response protein Heme oxygenase-1 (Ho-1) was increased in the liver \( (p = 0.017) \), while no significant changes were observed in the kidney \( (p = 0.068) \) in brain-dead versus sham animals (Fig S3B).
DISCUSSION

The aim of the present research was to evaluate the metabolic status of the brain-dead donor in order to explore novel methods to assess and target graft quality prior to transplantation. We demonstrated systemic, but also differentiated organ-specific, metabolic changes during BD. In the liver, we observed increased aerobic glycolysis with functional mitochondria, a shift towards fatty acid metabolism, and increased oxygen consumption compared to healthy controls. In the kidneys on the other hand, we found increased anaerobic metabolism concomitant with decreased renal perfusion, despite functional renal parenchyma that maintained normal oxygenation. Together, this represents a distinctive metabolic response in the brain-dead donor and provide a framework for further exploration of targeted donor and organ assessment and management strategies.

A systemic assessment of metabolic alterations following BD revealed changes in plasma metabolites that are similar to those observed during prolonged fasting and starvation, and that indicate glucose depletion, a shift towards fatty acid metabolism and a breakdown of amino acids. These observations confirm previous findings that demonstrated decreased glucose and increased fatty acid levels8,9 and that are comparable to those seen during prolonged fasting and starvation: the release of free fatty acids from fat tissue and their use as an alternative energy source when glucose levels are decreased10,11. Furthermore, lactate levels and blood pH were unaltered following BD, in agreement with a study on BD in pigs, in which lactate levels normalised within three hours of BD onset12. In contrast, clinical studies have shown variable lactate levels in brain-dead subjects13,14, as would be expected in heterogeneous situations. These variations in lactate levels may reflect differences in the ability to recycle lactate via the Cori cycle, a pathway that restores or maintains glucose levels during prolonged fasting and starvation in humans15.

Investigating organ-specific changes during BD, we showed that aerobic metabolism increased in the liver, with functional mitochondria and adequate hepatic perfusion. These results suggest that the liver remains metabolically active in order to meet the metabolic demands imposed by BD pathophysiology. Firstly, we showed decreased hepatic glycogen stores following BD, suggesting increased glucose mobilisation via gluconeogenesis. These results are in line with studies that showed increased levels of the glycolysis-regulating hormone glucagon in pigs during BD16. Secondly, gene expression data with in particular the increased expression of Pfk1, the key enzyme regulating the flux through glycolysis, suggests stimulation of glycolysis during BD. This induction of glycolysis combined with decreased ATP levels during BD supports the idea that the liver faces increased energy demands and responds by increasing glucose catabolism. Regulation of glucose metabolism is under direct control of catecholamines17, which stimulate aerobic glycolysis (and thus ATP production) and increase glucose release via gluconeogenesis and glucose homeostasis. Therefore, it is likely that the catecholamine surge during BD contributed to increased hepatic glycolysis and gluconeogenesis, even though we did not observe changes in gluconeogenesis. The absent gluconeogenic response in the liver following BD may be due to an experimental duration that was potentially too short to observe changes in gluconeogenic gene expression. Alternatively, the hepatic response to catecholamines may have been impaired due to changes in adrenergic receptor affinity, which can occur under pathophysiological conditions such as inflammation or bacteraemia18. Thus, BD-induced inflammation might have modulated the adrenergic and subsequent the gluconeogenic response in the liver.

However, the notion that the liver responds to increased energetic demands during BD is further supported by increased BOLD-related oxygen consumption, as well as the increased expression of metabolic proteins. BOLD MRI has been well established by researchers studying the central nervous and urogenital system to detect changes in oxygen availability. Initial exploratory studies in the liver have shown great promise of this technique in testing for therapeutic effects of tumour chemoembolization therapy16,17.

However, to ensure that the BOLD-related oxygen availability we observed was indeed a reflection of oxygen consumption, we assessed several factors also known to influence BOLD signalling, including changes in tissue blood flow, external factors including pH, hydration status, and effectiveness of oxygen consumption for example by the mitochondria16. Firstly, total hepatic blood flow, which normally represents 75% portal and 25% arterial flow18, did not change during the course of BD. Secondly, plasma pH and haemodynamic status did not differ between brain-dead and sham animals. Thirdly, mitochondrial function was not affected as was demonstrated by mitochondrial respiration and complex activity analyses. Taken together, these data suggest that increased BOLD-related signal (i.e. lower oxygen availability) is largely a reflection of increased oxygen consumption in the liver during BD. Moreover, we believe that this increased oxygen consumption can likely be attributed to increased metabolic activity. This is supported by the proteomics analysis showing increased mitochondrial protein expression of several peptides involved in the TCA cycle as well as FAO. The increased expression of proteins involved in these processes suggests that the liver facilitates increased oxidative metabolism during BD.

The adaptive hepatic response is in sharp contrast to the metabolic changes observed in the kidneys. Following BD, a shift towards renal anaerobic glycolysis is evident with decreased ATP levels and increased oxidative stress, which we attribute to decreased renal perfusion as evidenced by T1-weighted MRI. Normally, the kidneys are important players during prolonged fasting, maintaining glucose homeostasis by increasing gluconeogenesis19. However, following BD, renal expression of Pck-1, the key limiting enzyme of gluconeogenesis, was decreased in combination with increased anaerobic glycolysis and ATP depletion. Similar observations have been made in studies where renal blood flow was reduced following urinary tract obstruction20, as well as a study on renal ischemia in mice that showed reduced levels of cortical glucose, suggestive of decreased gluconeogenesis21. These results indicate that hypoxia and not an adaptive mechanism to prolonged fasting, influences metabolism in the kidney after BD.

The idea of hypoxia as a central injury mechanism is further supported by the decreased renal ASL signal, which suggests impaired renal perfusion during BD. The use of ASL as an alternative to contrast-dependent MRI has shown promise at detecting changes in renal perfusion and has positively been correlated to plasma biomarkers in renal allograft recipients during acute rejection22,23. In line with our results, a study on traumatic brain injury in rodents showed decreased renal blood flow 60 min after injury, visualised with radio-active microspheres, which the authors attributed to increased levels of circulating catecholamines24. As renal blood flow is under sympathetic as well as hormonal control25,26, it is conceivable that increased levels of circulating catecholamines, angiotensin II, and endothelin-1 during BD27 are responsible for the decrease in renal perfusion. This is in line with a study by Dibona et al. that showed decreased renal blood flow following stimulation of the renal sympathetic nerves, an effect that was absent following renal denervation28. Together these data suggest that the anaerobic changes in the kidney are caused by impaired perfusion, presumably mediated by increased levels of vasoconstrictive hormones or increased sympathetic stimulation.
Tissue perfusion of the kidneys is not the only determinant of oxygenation status, as it is also controlled by oxygen consumption, arterial-to-venous shunting, and oxygen saturation (SaO₂) levels⁴⁵. Firstly, we showed that oxygen availability and consumption, measured by mitochondrial respiration as well as BOLD MRI, were unaltered in the kidneys following BD. BOLD has previously been tested in transplanted kidney with acute rejection and tubular necrosis⁴⁶ and showed comparable results to invasive oxygenation measurements⁴⁷. These results suggest that the kidneys adequately consume and utilise oxygen following BD, portraying a metabolic phenotype similar to that of the diabetic kidney in so-called pseudo-hypoxic conditions⁴⁸,⁴⁹. In this condition, lactate production increases despite adequate oxygen consumption and mitochondrial function. Secondly, the observed hypoxic changes following BD are unlikely to be attributable to either changes in arterial-to-venous shunting or SaO₂. Shunting is believed to occur primarily as a protective mechanism during hyperoxia or severe hypoxia⁵⁰, and systemic SaO₂ levels were optimal throughout our experiment. Thus, our data suggest that the anaerobic changes in the kidney can be explained by decreased renal perfusion. Interestingly, a study on brain-dead rodents by Akhtar et al. suggested that mitochondrial function was impaired following BD, as demonstrated by impaired mitochondrial control ratios⁵¹. It is important to note that these results might be explained by a difference in experimental duration of the brain-dead versus the short, 30-min control group. An alternative explanation is that respiration was measured in cortical mitochondria alone⁵², which might suggest possible intrarenal differences in oxygenation and/or perfusion. Normally, perfusion and oxygenation is most predominant in the cortex of the kidney, while the medulla remains relatively hypoxic⁵³. However, to protect the sensitive medulla during hypoxia, blood flow can be redistributed in favour of the medulla⁵⁴. Another intrarenal difference is that vasoconstrictive hormones have a more pronounced effect on the cortex than the medulla⁵⁵. Therefore, we suggest that BD might affect cortical perfusion more than medullary perfusion, which might explain differences in mitochondrial function between these areas. Despite possible intrarenal differences or a redistribution of blood flow, our data suggest that the anaerobic changes and oxidative stress in the kidney following BD are the result of overall decreased renal perfusion.

We acknowledge that several limitations apply to this study. Due to the experimental setup sham animals were exposed to a longer anaesthetic duration compared to brain-dead animals, which introduced anaesthetic duration as a possible confounder. Fortunately, studies on the effects of sevoflurane administration in mice have shown that sevoflurane does not alter histopathology or function of the liver and kidneys⁵⁶,⁵⁷, suggesting that possible short-term effects of this anaesthetic are negligible. Furthermore, interpretation of our study results raised questions on possible intrarenal or intrahepatic differences in oxygenation, perfusion, and cellular function. Even though we are aware of the importance of these potential differences, exploring these differences was outside the scope of this study. Additionally, the use of isolated mitochondria to measure mitochondrial respiration may be disadvantageous, as cellular context and effects of proliferation and localisation are lacking⁵⁸. However, we have chosen to accept this risk as these statistical tests were performed for secondary outcome parameters (e.g. proteomics) which were used to indicate trends that were in support of our primary outcomes parameters (i.e. MRI and mitochondrial respiration).

In conclusion, we provide clear evidence that BD pathophysiology influences systemic metabolic processes, alongside organ-specific metabolic changes with noticeable differences between the liver and kidneys. The liver responds to higher metabolic demands by increasing aerobic metabolism with functional mitochondria, whilst facilitating the use of alternative energy sources. In contrast, the kidneys shut down metabolically and suffer from oxidative stress, shifting towards anaerobic energy production while renal perfusion decreases. These results highlight the need for an organ-specific approach to facilitate optimal function of the liver and kidneys following BD. We suggest that treatment of the liver graft should focus on raising metabolite supply with adequate oxygenation, thereby optimising the metabolic conditions for the liver. On the other hand, kidney function should be ameliorated by improvement of renal perfusion while supporting cellular detoxification, reverting back to aerobic metabolism and replenishing energy stores. An organ-specific, dual approach focusing on metabolic changes could be part of a new strategy to assess and treat organ grafts in the brain-dead donor or afterwards during preservation, and could be the key to improving transplantation outcomes.
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