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In preparation
ABSTRACT

Introduction: Organs from brain dead (BD) donors have worse long-term outcomes compared to living donors. Major metabolic changes have been observed in brain-dead organ donors. As metabolic changes can be related to primary mitochondrial dysfunction, our aim is to study mitochondrial function in the liver and kidney from BD animals.

Methods: BD was induced in 8 mechanically-ventilated rats by inflation of a Fogarty catheter in the epidural space. Sham-operated rats were used as a control group. Mitochondria from liver and kidney tissue were isolated by differential centrifugation. Oxygen consumption measurements were performed using a Clark type electrode, as well as mitochondrial proteomics and enzymatic activity assays.

Results: Small changes in oxygen consumption were found in liver and kidney isolated mitochondria. As an increase in the uncoupled control ratio was found in liver mitochondria from BD animals plus an increase in oxygen consumption during state 2 was found in kidney mitochondria from BD animals. An increase in uncoupling protein-2 was found in kidney and liver mitochondria. A decrease in complex I and ATP synthase activity was found in liver mitochondria of BD animals.

Conclusion: Brain death affects hepatic mitochondrial function by decreasing Complex I and ATP synthase activity, possibly caused by an increase in UCP2 present. However these changes are not affecting the majority of oxygen consumption measurements in kidney nor liver.
INTRODUCTION
The shortage of organs suitable for transplantation remains one of the major challenges in the field of organ transplantation. The use of extended criteria donors and deceased circulatory death (DCD) donors are common strategies used to address this problem. However, long- and short-term outcomes of grafts obtained from these sources are inferior compared to living donor-grafts [3, 40]. Interestingly, grafts from non extended criteria brain-dead donors show worse survival rates compared to HLA mismatched living donors [37–39].

Since donation after brain death (BD) continues to be the main source of organs for transplantation, research has focused on optimizing outcomes of transplantations with organs derived from brain-dead donors. Brain death can be understood as a complex disturbance in body homeostasis, resulting in hemodynamic instability [2, 10, 20, 41], hormonal impairment [9, 11, 21], and inflammation [1, 14, 23, 27]. The onset of BD is caused by significant and progressive cerebral ischemia. The resulting injury will lead to intracranial hypertension. Cushing described the sympathetic response to intracranial hypertension, which is also called catecholamines storm. This sudden increase in circulating vasopressors elevates blood pressure and can be considered the starting point of the hemodynamic pattern that characterizes BD. This pattern finishes with spinal cord ischemia and the loss of sympathetic tone in the peripheral vascular bed, triggering the vascular collapse at the end of the BD period [15, 30]. Hemodynamic disturbances and changes in the microcirculation can affect organ perfusion and tissue oxygenation, thereby altering metabolic status of the organs. In a BD model using carbon labeled compounds, Novitzky et al. [28] reported that brain dead animals showed increased anaerobic metabolism. This increase was accompanied by a deterioration of the hemodynamic status.

Another consequence of brain stem ischemia is hormonal dysregulation. As pituitary function is affected, plasma levels of cortisol, thyroid hormones (T₃/T₄), insulin, and antiuretic hormone decrease below baseline levels after BD has been established [4, 29, 30]. Thyroid hormones play an important role in metabolism, as a reduction in thyroid hormone levels causes a hypometabolic state. Yet, hormonal therapy is still matter of debate in current practice [7, 18, 33]. However, in previous experiments we showed that pre-treatment with 3, 3′, 5-triiodo-L-thyronine reduces apoptosis and improves organ quality in liver tissue of brain-dead rats.

BD is also characterized by a systemic inflammatory response, including a rise in circulating cytokines levels [23, 26, 39]. This inflammatory environment permits an influx of inflammatory cells in the kidneys, liver, and lung, triggering a local inflammatory and apoptotic response [14, 27]. The origin of this inflammatory status is not well understood, but there are reasons to assign a role to cerebral inflammatory cytokines, complement activation [5, 6] and increased intestinal permeability [16]. Our previous study shows that anti-inflammatory effects of prednisolone did not improve the quality of the kidney or liver of brain-dead rats [32].
These pathophysiological changes in the brain-dead donor cause a stressful environment with potential changes in organ perfusion, microcirculation and decreased hormonal regulation. Together, we believe that these changes are affecting organ metabolism during BD.

The metabolic status of donor organs has received more attention due to the current shift in organ preservation from the current standard of cold storage to hypothermic and normothermic machine perfusion. Yet, few articles have been written about the metabolic status of the brain-dead organ donors prior to transplantation. Sztark et al. [36] showed a shift to anaerobic metabolism and in muscle biopsies a mitochondrial dysfunction in BD donors. Mitochondria are essential players in cellular metabolism, in particular in their crucial role in providing nearly 90% of the required cellular energy by means of ATP synthesis.

Therefore, we aimed to evaluate mitochondrial activity by measuring oxygen consumption in isolated mitochondria from liver, and kidney of brain dead rats. Exploring possible metabolic changes in the donor can be crucial to improve donor treatment and ultimately organ quality for transplantation.

**METHODS**

**Animals**

Sixteen male, adult Fisher F344 (Harlan, UK) rats (250-300 g) were used. The Danish Inspectorate of Animal Experimentation approved the study. All animals received care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources and published by the Danish National Institutes of Health, Publication No. 86-23, revised 1996).

Animals were anesthetized using sevoflurane with 100% O2. Animals were intubated via a tracheostomy and ventilated using a Tidal Volume of 7 ml/BW (Kg) per stroke, a positive end expiratory pressure of 3 cm H2O, an initial respiratory rate of 120, and a base-corrected end-tidal CO2 throughout the experiment. Two cannulas were inserted in the femoral artery and vein for continuous mean arterial pressure (MAP) monitoring and volume replacement. Through a frontolateral hole drilled in the skull, a no. 4 Fogarty catheter (Edwards Lifesciences Co, Irvine, CA) was placed in the epidural space and inflated with saline using a syringe pump (Model 980-733, Harvard Apparatus, Harvard Bioscience Co., Massachusetts, US). A second hole was drilled contra-laterally for insertion of 24G cannula used for intracranial pressure (ICP) monitoring. Induction of BD was achieved by inflating Fogarty catheter at a speed of 16 ml/h. Inflation of the balloon was terminated once a rise in the MAP above 80 mmHg was noted, reflecting the autonomic storm at the beginning of BD. BD was confirmed by the absence of corneal reflexes and an ICP that superseded the MAP. Following confirmation of BD, ventilation was continued and anesthesia terminated. During the 4 h BD period, MAP was kept
above 80 mmHg, temperature around 37 degrees Celsius and EtCO₂ between 2.7 – 2.9 kPA. If necessary, colloid infusion with poly hydroxyethyl starch (HAES) 10% (Fresenius Kabi AG, Bad Hamburg, Germany) was started (at a maximum rate of 1 ml/hr) to maintain a normotensive MAP. Unresponsiveness to HAES administration indicated the start of an intravenous noradrenaline (NA) drip (1 mg/mL). A homeothermic heating control system was used throughout the experiment. For Sham operated animals the surgical procedure was the same except for insertion of the Fogarty catheter. Sham animals were anesthetized and mechanically ventilated for 4 h duration of the experiment. A Rocuronium (0.1 mg/ml at 0.3 ml/hr) drip was used in both sham and brain-dead animals to avoid sudden cramping during the experiment. A bolus of Rocuronium (0.1 mg) was also given at termination before aortic puncture.

After 4 h, blood and urine were collected, after which all abdominal organs were flushed with cold saline. After the flush-out, organs were harvested and tissue samples were snap-frozen in liquid nitrogen and stored at -80 °C or fixated in 4% paraformaldehyde. Plasma samples and urine were also snap-frozen and stored.

Animals were randomly assigned to one of the experimental groups (n = 8):
1. Brain Death
2. Sham

Mitochondrial Isolation
Mitochondria were isolated by differential centrifugation from the liver and kidney of male Fisher rats (275–300 g), procedure described in detail elsewhere [22]. After 4 h of BD, organs were quickly removed and placed into ice-cold isotonic (0.9%) KCl solution. The tissue was then weighed and about 1.5 g of liver and a whole kidney were cut into small pieces. The tissue was homogenized using a Potter-Elvehjem homogenizer with a medium containing 10 mM Tris, 250 mM sucrose, 3 mM EGTA, and 2 mg/ml bovine serum albumin (BSA) (pH 7.7, at 4 °C). Tissue homogenates were then centrifuged at 800 g for 10 min at 4 °C. Supernatant was then centrifuged at 7200 g for 10 min at 4 °C. The obtained pellet was washed in a buffer containing 250 mM sucrose and 5 mM Tris (pH 7.3, at 4 °C). The final centrifugation step was done at 7200 g for 10 min at 4 °C. The mitochondrial pellet was resuspended in 300 μL of the buffer used in the final centrifugation step. Protein content was determined using a BCA protein assay kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA).

Oxygen Consumption Measurements
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-Zk (Oroboros, Innsbruck, Austria). Assay medium contained 110 mM KCl, 20
mM Tris, 2.3 mM MgCl₂, 5 mM KH₂PO₄, and 1 mg/ml bovine serum albumin, pH 7.2. The oxidizable substrates were (i) 5 mM pyruvate + 2 mM malate, (ii) 5 mM succinate + 1 μM rotenone, (ii) 5 mM glutamate + 5 mM malate, (iv) 25 μM palmitoyl-Coa + 2 mM L-carnitine + 2 mM malate. Maximal ADP-stimulated oxygen consumption (state 3) was achieved by adding 4.8 U/ml hexokinase, 12.5 mM glucose and 1 mM ATP. Resting state (i.e. state 4) oxygen consumption rate was determined after blocking ADP phosphorylation with 1.25 μM carboxyatractyloside. Oxygen consumption rate in the uncoupled state (state U) was determined after addition of 2 μM Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP). Respiratory control ratio (RCR) was calculated by dividing oxygen consumption rate in state 3 by oxygen consumption rate in state 4. Uncoupled control ratio (UCR) was calculated by dividing oxygen consumption rate in state U by oxygen consumption in state 3. Data acquisition (4 Hz sampling frequency) and analysis were performed using DatLab software version 4.2 (Oroboros, Innsbruck, Austria).

Complex assay activity
Isolated mitochondria were used for enzymatic activity assays as described previously [13]. Mitochondria were diluted in PBS, lysed by sonification 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). The BSA standard solution was used for quality control. For Complex I activity, 2,6-dichloroindophenol (DCIP) was used as terminal electron acceptor. The reaction was followed spectrophotometrically at 600 nm at 37 °C in an assay medium with containing 25 mM potassium phosphate, 0.35% BSA, 70 μM decylubiquinone, and 1.0 μM antimycin A, by adding first 60 μM DCIP, then 0.2 mM NADH, and finally 1 μM rotenone. Complex I activity was calculated using the molar extinction coefficient of DCIP at 600 nm equal to 21000 M⁻¹cm⁻¹ (for path length of 1cm). Complex I activity is expressed as nmol/min/mg protein. For ATP synthase activity, the reaction was followed spectrophotometrically at 340 nm at 37 °C in an assay medium with containing 40 mM Tris, 5 mM magnesium chloride, 10 mM potassium chloride, 0.1% BSA, 2 U pyruvate kinase / 4 lactate dehydrogenase, 1.0 μM antimycin A, 3 μM carbonyl cyanide 4-trifluoromethoxy phenylhydrazone, by adding first 5 mM NADH, then 0.5 mM ATP and finally 4 μM oligomycin. ATP synthase activity was calculated using the molar extinction coefficient of NADH at 340 nm equal to 6220 M⁻¹cm⁻¹ (for path length of 1cm). ATP synthase activity is expressed as nmol/min/mg protein.

Targeted quantitative mitochondrial proteomics
Selected mitochondrial proteins involved in the substrate transport, fatty acid β-oxidation and TCA cycle were quantified in isolated mitochondria using isotopically labeled standards (¹³C-labeled lysines and arginines), derived from synthetic protein concatamers (QconCAT) (PolyQuant GmbH, Bad Abbach, Germany). Briefly, mitochondrial samples
(50 μg protein) were mixed with 1.5 ng of QconCAT per 1 μg of total mitochondrial protein. Then, reduced with 10 mM dithiothreitol and alkylation with 55 mM iodoacetamide. Proteins were subjected to in-gel tryptic digestion (1:100 g/g). Peptides were targeted and analyzed by a triple quadrupole mass spectrometer (MS) equipped with a nano-electrospray ion source (TSQ Vantage, Thermo Scientific). The chromatographic separation (gradient 100 min) of the peptides was performed by liquid chromatography on a nano-UHPLC system (Ultimate UHPLC focused, Dionex). The MS traces were manually curated using the Skyline software [19] prior to integration of the peak areas for quantification. The sum of all transition peak areas for the endogenous peptides and isotopically labeled QconCAT-peptide standards was used to calculate the ratio between the endogenous and standard peptides. The concentrations of the endogenous peptides were calculated from the known concentrations of the standards and expressed in pmol/mg of mitochondrial protein.

Statistical Analysis
For statistical analysis a Mann-Whitney test was performed, with p < 0.05 regarded as significant to compare between two groups. Results were presented as mean ± standard deviation (SD).

RESULTS
Mitochondrial function was determined using different substrate combinations - (i) 5 mM pyruvate + 2 mM malate, (ii) 5 mM succinate + 1 μM rotenone, (ii) 5 mM glutamate + 5 mM malate, (iv) 25 μM palmitoyl-Coa + 2 mM L-carnitine + 2 mM malate, by looking at oxygen consumption in different states (Figure 8.1). Isolated liver mitochondria from brain-dead animals had an increased uncoupled control ratio (UCR) when pyruvate + malate, succinate + rotenone, and glutamate + malate were used as substrates. A significant increase in oxygen consumption was found in state U as well as and the respiratory control ratio (RCR) using glutamate + malate (Figure 8.4). No differences were found in other respiratory states when pyruvate + malate (Figure 8.2), succinate + rotenone (Figure 8.3) and palmitoyl-Coa + L-carnitine + malate were used (Figure 8.5).

Isolated kidney mitochondria from brain-dead animals had an increased UCR when pyruvate + malate and glutamate + malate were used as substrates. An increase in oxygen consumption was found in state 2 using succinate + rotenone. No difference were found in other respiratory states when pyruvate + malate (Figure 8.6), succinate + rotenone (Figure 8.7), glutamate + malate (Figure 8.8) nor palmitoyl-Coa + L-carnitine + malate were used (Figure 8.9).

Complex I activity was measured in isolated liver and kidney mitochondria. Activity of Complex I in the liver was significantly reduced in brain-dead compared to sham animals.
Complex I activity in the kidney was not significantly different between groups (Figure 8.10C).

Activity of ATP synthase in the liver was significantly reduced in brain dead compared to sham animals (Figure 8.10B). ATP synthase activity in the kidney was not significantly different between groups (Figure 8.10D).

54 proteins were identified in isolated mitochondria from the liver and kidney. Proteins that significantly differed between brain-dead and sham animals are presented along with a short description in Table 8.1. Electron transport chain-related protein Cytochrome c-1 (Cyc 1) was present in significantly lower levels in liver of brain-dead animals compared to sham animals. Such difference was not observed in the kidney. NADH dehydrogenase (ubiquinone) Fe-S protein 1 (Ndufs1), a protein part of Complex I, had lower levels in kidney mitochondria of brain-dead compared to sham animals, but no changes were observed in the liver. Mitochondrial uncoupling protein 2 (UCP2) was higher expressed in both liver and kidney mitochondria in brain-dead animals versus sham animals. Lower levels of citrate cycle related proteins Aconitase hydratase (Aco2), Fumarate hydratase (Fh), and Succinate-CoA ligase (Suclg2) were found in kidney mitochondria of brain-dead compared to sham-operated animals. Interestingly, higher levels of fatty acid metabolism-related proteins Medium-chain specific acyl-CoA dehydrogenase (Acadm) and Very long-chain specific acyl-CoA dehydrogenase (Acadvl) were found in liver tissue of brain-dead compared to sham-operated animals. Finally, higher levels of Dihydrolipoyl dehydrogenase (Dld) were found in liver mitochondria of brain-dead compared to sham animals.

**DISCUSSION**

To explain lower graft quality Sztark et al. suggested that a primary metabolic dysfunction could be responsible for the global metabolic changes in BD donors [36]. They studied oxygen consumption in isolated mitochondria from permeabilized muscle fibers and showed mitochondrial dysfunction in human BD donors. Mitochondrial oxygen consumption is a reflection of the functionality of the electron transport chain and thus a functional parameter linked to mitochondrial functioning. However, the study of Sztark et al. gives no insights into mitochondrial function of the actual organs meant for transplantation, such as the kidney and the liver. In an attempt to investigate the effect of BD on the mitochondria in the donor organs, this study was designed to shed light on this matter.

In contrast to these previous findings, we did not find any global functional changes in the liver and kidney mitochondria of brain-dead animals. However, we did find interesting differences between liver and kidney. Liver mitochondria from BD animals portrayed a higher Uncoupled Control Ratio (UCR) using citrate cycle related substrates compared to sham-operated animals. This suggests that BD pathophysiology includes a higher degree of uncoupling of mitochondria in the liver of BD animals. A higher degree of uncoupling is in accordance with the higher amount of Uncoupling protein-2 (UCP2).
and lower amount of Cytochrome c-1 (Cyc1) that is present in these liver mitochondria. UCP2 belongs to a family of inner mitochondrial membrane proteins that are known for the ongoing discussion about their role in diabetes and cancer [25]. However, this family of proteins is also involved in reactive oxygen species (ROS) regulation [34, 35]. UCP2 limits ROS production by decreasing the electrochemical proton gradient across the mitochondrial inner membrane, and by reducing electron transport chain activity [17]. But it is also been discussed that UCP2 could act as a carrier and induces a metabolic shift enhancing the utilization of non glucose-derived substrates [8, 31]. We believe BD-induced ROS production increases UCP2 in the liver and kidney, resulting in uncoupling of the mitochondria. Uncoupling of the mitochondria in turn protects the organs for subsequent ROS-induced damage. Uncoupled mitochondria decrease their ATP production and simultaneously are able to enhance their oxygen consumption, as they are no longer limited by ATP synthesis. We indeed show that the significant increase in UCP2 protein levels in the liver and kidney mitochondria from brain-dead animals are associated with a higher UCR ratio and reduced ATP levels. Even more, this drop in ATP levels was accompanied by reduced ATP-synthase activity in liver mitochondria from BD animals compared to sham animals. In the kidney, uncoupling of the mitochondria, as reflected by a higher UCR and lower ATP levels is not accompanied by a decrease in ATP synthase activity.

Interestingly, other authors have failed to show this decrease in energy production in liver grafts from BD donors [12, 24]. We believe this can be attributed to the highly sensitive system we used to visualize these small yet significant changes in mitochondrial oxygen consumption and complex activities.

Besides ATP synthase activity, BD also causes a reduction in Complex I activity in liver mitochondria compared with sham-operated animals. However, this deficiency in Complex I in the liver was not observed when we compared oxygen consumption with substrates that bypass Complex I, like succinate + rotenone, to substrates that do not, like pyruvate + malate. As such, we did not find a functional reflection of this deficiency in oxygen consumption measurements nor difference in protein expression to explain this difference in Complex I activity. However, mitochondrial uncoupling could bypass Complex I and kept oxygen consumption stable.

Liver mitochondria from BD animals also show increased oxygen utilization in the presence of fatty acid and amino acid metabolites, alternatives to carbohydrate metabolites. Thus, these differences could reflect a change in fuel source during BD, caused by an increased energy demand and a decrease in carbohydrates availability. However, a much deeper understanding into the various metabolites and enzymes involved is required to understand this possible shift.

Kidney mitochondria from BD animals show increased substrate drive oxygen consumption using citrate cycle related substrates, contrary to our results in protein expression that show a decrease in the presence of enzymes related with citrate cycle.
Moreover Complex I and ATP synthase activity is not affected in BD animals compared to sham-operated animals. This indicates that even though renal mitochondrial functioning is slightly altered in the BD setting, this alone might not explain the drop in renal ATP levels following BD. As we know from previous experiments (see Chapter 7), kidney perfusion is lower in BD animals compared to sham-operated animals. Therefore, this suggested that this decrease in energy production in kidneys from brain dead animals is closely related with a lack of oxygen delivery more so than a primary mitochondrial dysfunction.

In conclusion, these changes in oxygen consumption, expression of UCP-2 and decreased activity of Complex I and ATP synthase in liver but not in kidney isolated mitochondria highlight interesting differences in the response of the kidney and liver to BD condition. It also shows the different conditions that these organs face before transplantation and the need for a two-pronged approach to treat a brain-dead donor. The approach to ameliorate kidney function should focus on improving kidney perfusion during BD or afterwards in an isolated machine perfusion device. Simultaneously, treatment of liver grafts should be focused on improving protection against ROS and raising fuel supply to these grafts. A dual approach could be key in the treatment of brain-dead organ donors and improving organ quality of marginal grafts.
REFERENCES


FIGURES

Figure 8.1: Representative schema of oxygen consumption states using Clark-type electrodes.

Figure 8.2: Oxygen consumption in isolated liver mitochondria using pyruvate and malate as substrates. Mitochondrial function in Brain Dead Rats.
Figure 8.3: Oxygen consumption in isolated liver mitochondria using succinate and rotenone as substrates.
Figure 8.4: Oxygen consumption in isolated liver mitochondria using glutamate and malate as substrates.
Figure 8.5: Oxygen consumption in isolated liver mitochondria using palmitoyl-CoA, L-carnitine and malate as substrates.
Figure 8.6: Oxygen consumption in isolated kidney mitochondria using pyruvate and malate as substrates.
Figure 8.7: Oxygen consumption in isolated kidney mitochondria using succinate and rotenone as substrates.
Figure 8.8: Oxygen consumption in isolated kidney mitochondria using glutamate and malate as substrates.
Figure 8.9: Oxygen consumption in isolated kidney mitochondria using palmitoyl-CoA, L-carnitine and malate as substrates.
Figure 8.10: Enzymatic activity of ATP synthase and Complex I from isolated liver and kidney mitochondria.
# TABLES

Table 8.1: Proteomics mitochondria data summary

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<td>Liver</td>
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### Table 8.1: (continued)

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