Impact of Brain Death on Donor Kidneys: Early Progression of Endothelial Activation, Oxidative Stress and Tubular Injury

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Abstract

Cerebral injury leading to brain death (BD) causes major hemodynamic instabilities in potential organ donors that may induce endothelial activation and affect post-transplant graft function. We investigated pro-coagulatory and pro-inflammatory effects of endothelial activation after BD with the subsequent oxidative stress and renal tubular injury.

Brain death was induced by slowly inflating over a period of 30 minutes of a balloon-catheter inserted in the extradural space. To assess time-dependant changes due to BD, rats (n=30) were sacrificed 0.5, 1, 2, or 4 hours after BD-induction and compared to sham-operated controls. E- and P-Selectins, fibrinogen mRNA were abruptly and progressively up-regulated from 0.5 hours BD onwards; P-Selectin membrane-expression was increased. Plasma von Willebrand factor was significantly higher after 2 hours and 4 hours BD, reflecting sustained platelet adhesion to the vascular-wall. Oxidative stress in kidneys was detectable only late, being significantly increased in 2 hours, and 4 hours groups. Urine heart–fatty–acid–binding–protein and N–acetyl–glucosaminidase, used as new specific and more sensitive markers of proximal and distal tubular damage, were found to be significantly increased after 0.5 hours, and maximum at 4 hours.

This study demonstrates immediate pro-coagulatory and pro-inflammatory activation of vascular endothelium after BD in kidney donor rats, proportional with the duration of BD. Oxidative stress measurements pointed at ischemia/reperfusion injury during protracted periods of BD. BD-related donor kidney damage was diagnosed after half hour of BD.
6.1 Introduction

To date, the retrieval of kidneys in brain death donation is primarily dependent on the logistics concerning the donor operation and the timing of the donor retrieval team. Following previous work that clearly documented the detrimental effect of a prolonged state of brain death, the clinicians recognize more and more the need to retrieve organs as soon as possible, avoiding unnecessary prolongation of organ procurement, in order to maintain viability.

Due to cerebral injury with subsequent brain death and loss of integrated neurological function, the potential organ donor is exposed to major physiologic derangements\textsuperscript{1–3}. To maintain function, an aggressive, labor-intensive donor management is required throughout the ICU stay.

Endothelial activation in brain dead donors has gained lately considerably attention in the discussion concerning the pathological effects of brain death on donor organ quality prior to retrieval. The vascular endothelial phenotype is known to change dramatically under various pathophysiologic conditions, expressing cell adhesion molecules, releasing cytokines and substrates that promote thrombosis and inflammation. Under hemodynamic and rheological unstable conditions endothelial cells have demonstrated their ability to sense variations in mechanical forces such as shear stress, that appear as a consequence of blood flow and viscosity alterations\textsuperscript{4,5}. Studies investigating the response of flow-adapted endothelial cells have shown that variation in shear stress was followed within minutes by membrane depolarization, increased intracellular Ca\textsuperscript{2+}, release of nitric oxide, reactive oxygen species, von Willebrand factor, prostacyclins, tissue factor, tissue plasminogen activator, adhesion molecules and chemoattractant proteins\textsuperscript{6,7}. Previous studies suggest that an immune activation with increased endothelial cell activation and immediate early gene expression occurs after brain death induction\textsuperscript{8–10}. Moreover, the expression of endothelial adhesion molecules (intercellular adhesion molecule–1 and vascular cell adhesion molecule–1) and the influx of leukocytes in the kidney is shown to occur faster and be more profound when hemodynamic instability in the brain dead donor is not corrected\textsuperscript{11}. Furthermore, the non-specific inflammatory response activated during brain death was shown to accelerate acute rejection of organs procured from brain dead donors\textsuperscript{12}.

As an original contribution, this study aims to analyze and document the time sequence for the most early progression post–BD induction of pro–inflammatory and pro–coagulatory endothelium activation, oxidative stress and organ viability in brain dead rat kidney donors. We hypothesized that activated endothelium in the brain dead donor will express and release both pro–inflammatory and pro–coagulatory factors into circulation that will mediate inflammation, platelet adhesion and possibly promote microthrombosis. In addition, we expect that due to activation of endothelium and hypoxic stress, the oxidative stress and brain death–related organ dysfunction will arise early after BD–induction.
6.2 Methods

**Animals and Experimental protocols**

The experiments were in accordance with institutional and legislator regulations and approved by the local Committee for Animal Experiments. A total of 30 rats (adult male Fisher 344 rats, 260–300 g, Harlan, Zeist, The Netherlands) were studied. To assess time–dependant changes due to brain death (BD), the animals (n=6 per group) were sacrificed after 0.5, 1, 2, or 4 hours after induction of BD. Controls (n=6) consisted of sham–operated rats using a trepanation, however without inserting the balloon catheter to cause cerebral injury. Sham–operated rats remained ventilated and under anesthesia for half hour. All rats were sacrificed after completion of the experiment.

**Surgical procedures**

Animals were anesthetized using oxygen/nitrous oxide/isoflurane 5%; isoflurane was reduced to 2% after anesthesia induction. Corporeal temperature was maintained at 37°C. After frontolateral trepanation lateral of the bregma, a balloon catheter (0.75 ml 4F EMB, Edward Lifesciences ref 120404F) was inserted and slowly inflated over a time period of 30 min with 0.5 ml water using a syringe pump. After approximately 27 min., the rats became apneic and were mechanically ventilated (12–15 mmHg relief, 1–5 mmHg positive end–expiratory pressure, Zoovent CWC600AP; Triumph technical services Ltd, United Kingdom.) through a tracheostoma (47/min frequency, 40% inspiration phase). After BD induction, anesthesia was stopped and the rats were ventilated with 100% O₂ for 30 min; subsequently ventilation was switched to O₂/air. Ten minutes before retrieval of organs, the rats were ventilated with oxygen/nitrous oxide/isoflurane 0.5% to allow muscle relaxation and laparotomy. Brain death was confirmed by the absence of brain stem reflexes, the pupillary reflex, the corneal reflex and an apnoea test. The mean arterial pressure was continuously measured and recorded using an intra–arterial blood pressure sensor (Truwave, Edwards Lifesciences, Irvine, USA, recorder Labview 5.1; National instruments Co., Austin, USA). A MAP lower than 80 mmHg was corrected by colloid infusion (10% hydroxyethyl starch, HAES, 37°C). Kidneys were retrieved after a flush with saline through the abdominal aorta, snap frozen in liquid nitrogen and stored at –80°C. To study the pro–inflammatory response as a result of the induction of brain death we investigated endothelial gene expression of E– and P–Selectin (real time RT–PCR), and membrane expression of P–Selectin (immunohistochemistry). The pro–coagulatory response during brain death was assessed by investigating circulating levels of von Willebrand factor (ELISA) and fibrinogen gene expression.
**Immunohistochemistry**

P–Selectin staining (Table 6.1) was performed on cryosection, acetone fixed slides. A semi–quantitative evaluation of the P–Selectin endothelial expression was performed in a double–blind fashion by two independent pathologists in parallel. The semi–quantitative scoring system used for grading P–Selectin endothelial staining had a scale of 0 to 3 arbitrary units: 0 (none), 1 (mild), 2 (moderate), 3 (intense).

In addition, a P–Selectin positive platelets counting was performed, considering each time 30 randomly chosen glomeruli per slide and reporting the results as average platelet number per glomeruli.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Type</th>
<th>Dilution</th>
<th>Incubation time</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>primary antibody</td>
<td>rabbit polyclonal antibody</td>
<td>1:25</td>
<td>1 hour</td>
<td>BD Pharmigen</td>
</tr>
<tr>
<td>against rat P–Selectin</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>2nd antibody</td>
<td>1:100</td>
<td>30 min</td>
<td>Vector Laboratories</td>
</tr>
<tr>
<td>goat anti–rabbit</td>
<td></td>
<td></td>
<td></td>
<td>BA–1000</td>
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<tr>
<td>immunoglobulin antiserum</td>
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<td>peroxidase conjugated</td>
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<td>BA–1000</td>
</tr>
<tr>
<td>rabbit anti–goat polyclonal antibody</td>
<td></td>
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</tbody>
</table>

Table 6.1: Immunohistochemical staining of P–Selectin on cryostat section, acetone fixed slides of rat kidney tissue. Antibodies were diluted in PBS containing 1% bovine serum albumin; 1% normal rat serum was added to the secondary antibodies. The peroxidase activity was developed using 3–amino–9–ethylcarboxide (AEC)/H$_2$O$_2$.

**Real time reverse transcriptase PCR** for Aα and Bβ fibrinogen chains, E– and P–Selectin gene expression were assessed using amplification primers designed with Primer Express software (Applied Biosystems, Foster City, USA). The primers sequence and product sizes are included in Table 6.2. Amplification and detection were performed with an ABI Prism 7900–HT Sequence Detection System (Applied Biosystems, Foster City, USA) using emission from Sybr green. All assays were performed in triplicate. Gene expression was normalized with the mean of β–actin mRNA content and calculated relative to controls using the relative standard curve method. Results were finally expressed as $2^{-\Delta C_T}$ ($C_T$ threshold cycle).

Plasma vWF concentrations were measured by ELISA (Coamatic von Willebrand Factor kit, Nodia BV, Amsterdam, The Netherlands).

**Detection of oxygen radicals production**

A fluorophore–nitroxide was used to image oxygen radicals generated during ex–vivo incubation of kidney biopsies$^{13–15}$. Both superoxide and hydroxyl radicals were con-
Verted and measured as H$_2$O$_2^{16}$. Fluorescence was measured with a multilabel counter (390 nm excitation and 510 nm emission filters, Victor2, EG&G Wallac, Turku, Finland). Standard curves were obtained by adding known amounts of H$_2$O$_2$ to the assay medium. A separate standard curve was prepared including stepwise diluted hemoglobin. The linear relationship between hemoglobin concentrations and fluorescence signal was used to correct for the hemoglobin signal quenching. Hemoglobin concentration in the supernatant of the incubated biopsies was measured by the method of Harboe$^{17}$. H$_2$O$_2$ concentration were corrected for the dry weight of the biopsy.

### Kidney injury biomarkers

Urine heart–type fatty acid binding protein (H–FABP) – ELISA (HyCult Biotechnology B.V., Uden, The Netherlands). The kit has a minimum detection limit of 0.4 ng/ml and a measurable concentration range of 0.4–25 ng/ml. Samples were diluted 10 times before measurement.

Urine N–acetyl–glucosaminidase (NAG) – modified enzyme assay according to Lockwood$^{18}$ at pH 4.5 and corrected for non–specific conversion (HaemoScan, Groningen, The Netherlands).

Both NAG and H–FABP urine concentrations were corrected for dilution using urine creatinine values.

### Statistical Analysis

The statistical analysis was performed using SPSS (Statistical Package for the Social Sciences).

Before analysis, the data was tested for distribution according to Kolmogorov–Smirnov goodness of fit test. To investigate differences between groups, continuous variables were compared by means of parametric (Student T Test) or nonparametric tests (Mann–Whitney). A p value smaller than 0.05 was considered statistically significant. Results are presented as mean±SEM (unless stated otherwise).

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### Table 6.2: Primers sequence and product sizes used for Real time reverse transcriptase PCR for Aα and Bβ fibrinogen chains, E– and P–Selectin gene expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequences</th>
<th>Reverse primer sequences</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aα fibrinogen</td>
<td>5'-GCTCTGTGCCAGGGTTGAATTA-3'</td>
<td>5'-GCCTACCCCGGAAGTGACTTC-3'</td>
<td>73 bp</td>
</tr>
<tr>
<td>Bβ fibrinogen</td>
<td>5'-CGGGCGGCTGGTATATACG-3'</td>
<td>5'-CTGTAAGGCACCCCACTGATAT-3'</td>
<td>71 bp</td>
</tr>
<tr>
<td>E–Selectin</td>
<td>5'-GTCTGCGATGCTGCCTACTTG-3'</td>
<td>5'-CTGCCACAGAAAGTGCCACTAC-3'</td>
<td>73 bp</td>
</tr>
<tr>
<td>P–Selectin</td>
<td>5'-TCTCTGGGTCTTCACTGATCTTCT-3'</td>
<td>5'-GTGTCCTTCCCTAGTACCCTATGAA-3'</td>
<td>71 bp</td>
</tr>
</tbody>
</table>
6.3 Results

**Hemodynamics.**

The blood MAP measured prior to balloon inflation was $116\pm3\text{mmHg}$. After $10\pm1$ minutes from inflation, the blood pressure decreased sharply and remained in a hypotensive state ($59\pm2\text{mmHg}$) for another $11\pm1$ minutes. At the end of the brain death induction the recording showed a sharp peak ($142\pm5\text{mmHg}$). $10.31\pm0.90$ minutes after the onset of brain death the blood pressure decreased to $46\pm1\text{mmHg}$. Subsequently, a peak in blood pressure occurred, followed by a plateau at levels above $100\text{mmHg}$ (Fig. 6.1).

Basal heart rate was $356\pm8\text{beats/minute (bpm)}$. During inflation of the balloon a slight increase in heart rate was observed. After the peak in MAP had occurred, a decline in heart rate to its basal level was observed.

**Donor management.**

During mechanical ventilation the MAP remained at levels above $80\text{mmHg}$. Five animals needed colloid infusion ($3\text{ml/kg}$) to correct for hypotension. All five responded well with a return to basal levels within three minutes. Thirty minutes after BD induction, the apnea test was found positive for all animals, with no spontaneous respiration. Corneal and pupillary reflexes were absent as well.

![Figure 6.1: Mean arterial pressure (MAP) monitoring during and after progressive brain death induction. The values are represented as mean (symbols) and standard error of the mean (bars).](image)
Organ Viability in Brain Dead Donors

Plasma von Willebrand factor (Fig. 6.2)
In the 0.5 h BD and 1 h BD groups, plasma vWF values remained in the normal range or increased moderately but only in sporadic cases. After 2 h of BD, however, vWF increased sharply, reaching significant different values in the 2 h BD (207±52%) and 4 h (248±88%) groups as compared with controls (Mann–Whitney p=0.002 for both time points).

Fibrinogen mRNA expression in rat kidney tissue (Fig. 6.3a,b)
Both Aα and Bβ fibrinogen chain mRNAs expression in renal tissue were highly and significantly up-regulated after 2 hours (Aα fibrinogen 22±12.9 fold induction, Mann–Whitney p=0.01; Bβ fibrinogen 7.8±4.6 fold induction, Mann–Whitney p=0.016) and 4 hours (Aα fibrinogen 86±36 fold induction, Mann–Whitney p=0.004; Bβ fibrinogen 54.6±29.6 fold induction, Mann–Whitney p=0.004) of brain death.

E-Selectin mRNA expression in rat kidney tissue (Fig. 6.4a) was up-regulated early, reaching already at 0.5 h post-BD folds ten times higher (10±3.5, Mann–Whitney p=0.004) than control values. The expression continued to increase in time, so
Figure 6.3: Relative gene expression (mRNA fold induction on a logarithmic scale) of A\(\alpha\) (a) and B\(\beta\) (b) fibrinogen chains in the kidney tissue of brain-dead rats after 0.5, 1, 2, or 4 hours since brain death (BD) induction. The controls are represented by sham-operated animals. Box plots graph data represent statistical values (see Fig. 6.2).

that the relative gene expression was 13.6±3.7 (fold induction) at 1 h of BD (Mann–Whitney p=0.002). After 2 h of BD the E–Selectin gene was strongly up–regulated (54.7±14.2 fold induction, Mann–Whitney p=0.002). The animals brain dead for 4 h had the most important up–regulation in E–Selectin gene expression (135.9±56.2 fold induction, Mann–Whitney p=0.002).

**P–Selectin mRNA expression in rat kidney tissue (Fig. 6.4b)** was significantly up–regulated starting with 1 h of BD (12.4±3.1 fold induction, Mann–Whitney p=0.002). P–Selectin gene expression increased progressively starting with 2 h BD (58.9±17.6 fold induction, Mann–Whitney p=0.002), with maximum values at 4 h BD (92.4±26.8 fold induction, Mann–Whitney p=0.002).

**P–Selectin Immunohistochemistry (Fig. 6.5a,b)**

P–Selectin endothelial membrane expression was absent in control samples (Fig. 6.5a), while in BD animal samples it started to be observed as early as half–hour after BD induction. The expression continued to increase, so that at 4 hours BD P–Selectin was omnipresent on the surface of vascular endothelial cells in the renal tissue (Fig. 6.5b). The arbitrary P–Selectin expression score at 4 h BD (2.2±0.2) was significantly higher (Mann–Whitney p=0.004) than the score in controls (0.33±0.22). Besides en-
dothelial expression, P–Selectin was stained in platelets trapped in the glomeruli, after washing–out the organs. After 1 hour of BD the platelet count per glomeruli (3.09±0.6 platelets/glomeruli) was significantly higher (Mann–Whitney p=0.04) that the number of platelets trapped in the controls samples (0.96±0.6 platelets/glomeruli).

**Oxidative stress (Fig. 6.6)**

H₂O₂ production in the kidney tissue increased non-significantly during the first hour of brain death (0.5 h BD 18.6±9.6; 1 h BD 51.6±21.4). H₂O₂ values became significantly higher than control values after 2 h BD (115.7±32, Mann–Whitney p=0.004). After 4 h BD, H₂O₂ production (401.7±95, Mann–Whitney p=0.004 vs. controls) reached values about 30 times higher than control values and 8 times higher than the values at 1 h BD.

**Tubular renal injury (Fig. 6.7a,b)**

Heart–type fatty acid binding protein (H–FABP), was below detection limits in plasma of all animals, showing minimal release into the circulation of this protein from heart, skeletal muscle, lungs, and brain. H–FABP was also below detection limits in the urine of sham–operated animals. Urine H–FABP concentrations started to rise above detection limits to as early as 0.5 h BD (14.2±5.06 ng/mmol creatinine) and continued to increase after 1 h BD (21.6±5.4 ng/mmol creatinine). Urine H–FABP concentra-
tions at 2 h and 4 h BD were 51.3±7.8 ng/mmol creatinine, and 52.6±8.8 ng/mmol creatinine respectively.

\(N\)-acetyl–glucosaminidase (NAG) urine concentrations started to increase non–significantly after 0.5 h of BD, reaching significant higher values 1 h after BD induction (4.2±1 mU/mmol creatinine, Mann–Whitney p=0.01) when compared with control values. Urine NAG continued to increase, reaching the maximum at 4 h BD (2 h BD 6.6±0.2 mU/mmol creatinine, Mann–Whitney p=0.002 vs. controls; 4 h BD 10±2.3 mU/mmol creatinine, Mann–Whitney p=0.002 vs. controls). Urine H–FABP and NAG concentrations correlated significantly (Spearman’s correlation coefficient 0.733, sig.<0.001).

6.4 Discussion

The present study demonstrates that brain death induces immediate pro–inflammatory and pro–coagulatory activation of vascular endothelium in rat donor kidneys, which is proportional with the duration of brain death. BD–related donor kidney damage and oxidative stress became subsequently evident, with enhanced injury with prolongation of the BD state. For this study we have used a simple, reproducible and clinical relevant animal brain death rat model in which induction of brain death was obtained by gradual expansion of an intracranial balloon over 30 minutes time period\textsuperscript{19}. The model represents an adjustment of brain death models published before by Tilney et al.\textsuperscript{20}, where the brain death induction was performed over a period of 15 minutes. The model described here is closely related to the clinical condition of BD due to intracranial haemorrhage, nowadays the most frequent diagnosis of organ donors. This approach stands in contrast to previous studies by our group and by others, where brain death was induced using an explosive onset model with massive brain destruction and critical hypotensive periods reflecting major head trauma. The major benefit of pseudo–stable hemodynamics in the brain death period is that no inotropic medical support is required, which is known to have an effect on organ injury and could bias results in studies concerning brain death related organ damage. Because intracranial hypertension often develops gradually in the clinical setting, we feel that the clinical situation is better represented by this model.
Figure 6.5: Immunohistochemistry using P-Selectin labelled antibody to stain (brown coloration) activated endothelial cells and trapped platelets in rat kidney tissue of a sham-operated animal (a) and a 4 hours brain death animal (b).
Figure 6.6: Oxidative stress, as quantified by ex–vivo $H_2O_2$ generation in rat kidney tissue after 0.5, 1, 2, or 4 hours since brain death (BD) induction. The controls are represented by sham–operated animals. Box plots graph data represent statistical values (see Fig. 6.2).

Figure 6.7: Renal tubular injury, as quantified by heart–type fatty acid binding protein (H–FABP, distal tubular injury, (a) and N–acetyl–glucosaminidase (NAG, proximal tubules injury, (b) in the urine of rats after 0.5, 1, 2, or 4 hours since brain death (BD) induction. The controls are represented by sham–operated animals. Box plots graph data represent statistical values (see Fig. 6.2).
Hemodynamics

After the onset of brain death the blood pressure decreased to 45 mmHg, most likely due to changes in the vasomotor tone caused by an imbalance between sympathetic and vagal stimulation of the rostral ventrolateral medulla. A gradual increase in blood pressure was observed afterwards, considered a physiological response (Cushing response) to increased intracranial pressure. Subsequently, a peak in blood pressure occurred, followed by a plateau at levels above 100 mmHg. A possible explanation for pseudo-normotension instead of the frequently reported hypotension is that a gradually expanding intracranial mass allows the brain to accommodate, with less distortion in the remaining rostral ventrolateral medulla.

Recent studies reported strong evidences of causality between hemodynamic impairment and the systemic inflammatory response. Even if the mechanisms remain unknown, Avlonitis et al. concluded that the sympathetic discharge triggered systemic inflammation, which was further enhanced by neurogenic hypotension.

The endothelial wall has demonstrated abilities to sense small hemodynamic, rheologic and humoral variations with prompt responses to various mechanical (shear stress, viscosity) and inflammatory (interleukins, TNF–α) stimuli. Once activated, the endothelial cells change phenotype to release and synthesize on-demand several vasoactive factors and factors involved in hemostasis and thrombolysis, such as nitric oxide, prostacyclins, vWF, tPA, tissue factor, adhesion molecules and chemoattractant proteins.

In our study, we approached endothelial activation by studying the acute release of von Willebrand factor, and expression of E– and P–Selectin. As an additional indicator of increased thrombogenicity we studied the gene expression induction of Aα and Bβ fibrinogen chains. Oxidative stress and kidney injury biomarkers were included in the investigation in order to quantify the clinical relevant end–effect of brain death on organ viability.

Von Willebrand Factor (vWF)

Von Willebrand Factor, stored in the endothelial Weibel–Palade storage granules, has unique biomechanical properties and a critical biological role as an adhesive protein; it mediates the adhesion of platelets to injured vascular wall by binding on platelet surface and to collagen in the subendothelium. vWF is one of the most potent activators of platelets, causing them to release additional vWF from their α–storage granules. Increased levels of plasma von Willebrand factor contribute directly to thrombosis, impeding the normal flow of circulating blood.

Allograft survival, arteritis and irreversible acute or sub–acute rejection have been reported to be highly associated with intensive staining for vWF on endothelial cells and platelets aggregating in large, medium and small arteries. Furthermore, increased plasma vWF represents a major risk factor for atherosclerosis and vascular disease. It has to be considered as a potential predictor for the development of the alloatherosclerosis of donor organ vessels and chronic rejection through endothelial
injury–induced proliferation of smooth muscle cells.

In our experiment, plasma von Willebrand factor started to rise prominently and significantly two hours after brain death induction, with maximum values after four hours of brain death in this rat model. The elevation in plasma level was significant, reaching levels of more than two times higher than in sham–operated controls.

**Fibrinogen**

Fibrinogen is a plasma protein whose principal function is exerted through its conversion into soluble fibrin during the process of blood coagulation. In addition, by virtue of its capacity to support platelet aggregation, fibrinogen plays a dual role in thrombus formation. Historically, fibrinogen is known to be synthesized exclusively by hepatocytes and stored in α–granules of megakaryocytes\(^ {27,28} \). In light of the recently published studies, it is clear that epithelial cells of extrahepatic origin are able to express fibrinogen genes and to secrete intact fibrinogen. Baumheuter et al.\(^ {29} \) found that in addition to being present in the liver, fibrinogen can be expressed on epithelial cells in the kidney, intestine, and spleen. Haidaris\(^ {30} \) demonstrated that, while hepatocytes synthesize and secrete fibrinogen constitutively and on demand, lung epithelial cells synthesize and secrete little intact fibrinogen constitutively. However, after induction with proinflammatory mediators, significant levels of fibrinogen are synthesized and secreted.

Our experiments conclusively demonstrate the expression of both Aα and Bβ fibrinogen chains in the kidneys of brain dead rats. Aα and Bβ fibrinogen chain mRNAs, while poorly expressed in kidneys of sham–operated rats, were abruptly and progressively up–regulated from two hours of brain death onwards, providing evidence that kidneys contribute to changes in acute phase proteins during brain death. The synthesis of fibrinogen in extrahepatic tissue may be triggered in the context of a systemic inflammatory response to brain death. Furthermore, fibrinogen up–regulation might attempt to restore homeostasis by contributing to wound repair or extracellular matrix remodelling after injury.

**E– and P–Selectins**

E– and P–Selectins belong to the Selectin family of adhesion molecules and play an important role in the inflammatory response, eliciting leukocyte rolling. Both P– and E–Selectins are reported to be critically involved in the early development of acute graft rejection\(^ {31} \). Up–regulation of gene expression and membrane E– and P–Selectin expression was described before in models of explosive brain death induction, and in recipients of transplanted grafts from brain dead donors\(^ {32,33} \). A P–Selectin positive expression in a donor biopsy present before transplantation has been shown to predict a high risk of acute rejection\(^ {34} \).

Complementary to the existing data on E– and P–Selectins in brain dead donors, we show an abrupt and progressive up–regulation of E– and P–Selectins starting very
early in the course of brain death, already after half hour from induction and persisting until four hours later. As verified by immunohistochemistry, mRNA up–regulation was closely followed by endothelial cells membrane expression of synthesized selectins. P–Selectin was detected on the surface of endothelial wall starting with half hour of brain death. The intensity and distribution of the staining increased with time, so that in the four hours brain death group P–Selectin was ubiquitously present on the surface of the vascular endothelium. In addition to expression on endothelial cells, P–Selectin labelling revealed the presence of an increased number of platelets in the kidney glomeruli. Since all organs were washed–out consistently and in a standardized manner, a progressively increasing number of platelets in time might reflect enhanced platelet adhesion to the vascular endothelium. These findings are consistent with the data showing increased plasma vWF levels in brain dead rats, a factor known to support platelet adhesion to the vascular wall.

Clinical and experimental studies investigating the therapeutic role of administrating recombinant proteins targeted against P–Selectin showed a marked improvement in graft outcome, blocking neutrophil and lymphocyte infiltration and thus decreasing inflammatory response and ischemia–reperfusion injury\(^{35,36}\). Different therapeutic approaches were studied, administrating the P–Selectin blocker to the donor (after 6 h BD–kidneys perfused in situ, or 3 h BD–intravenous injection) or to both donor and recipient (after 3 h BD intravenous injection to the donor and at the time of reperfusion in the recipient). The results of the present investigation show E– and P–Selectin up–regulation and expression already after half hour from brain death declaration, pointing out the need to introduce blocker therapy a lot earlier in the course of brain death donation.

**Oxidative stress**

Different authors have found reactive oxygen species (ROS) formation to be a reliable predictor of allograft rejection, especially in the early ischemic post–transplant period\(^{37}\). High levels of ROS are also known to contribute to the development of atherosclerosis of organ donor vessels and chronic rejection through endothelial–injury–induced proliferation of smooth muscle cells\(^{38}\).

In our approach, we have tested ex–vivo the mitochondrial (dys)function in kidney tissue of brain dead animals, as reflected by ROS–production when oxidizing pyruvate and succinate. General evidence for the source of ROS production points at dysfunctional mitochondria in cells pre–exposed to hypoxia–reoxygenation, activated endothelial cells and infiltrated inflammatory cells\(^{39–41}\). The production of superoxide and hydroxyl radicals, measured as \(H_2O_2\) generation, started to increase one hour after BD induction, reaching levels significantly higher only two hours after brain death induction, and maximum values after four hours. It is important also to analyze these results in the time sequence presented here: oxidative stress arises only after the pro–inflammatory response during brain death. This observation might solve a very
important mechanistic issue – it is the inflammatory response and not the hypoxia that is triggered first after brain death!

**Kidney injury (bio)markers**

To date, sensitive and specific biomarkers to assess donor organ viability are lacking. Non–invasive and easy accessible biomarkers assessing renal tubular injury are heart–type fatty acid binding protein (distal tubular injury) and N–acetyl–glucosaminidase (proximal tubular injury). Both markers were included in this study to investigate BD–derived renal damage.

**Heart–type fatty acid binding protein (H–FABP)** is a cytosolic protein abundant in the myocardium, but also expressed in kidneys (distal tubules), skeletal muscles, lung, and brain\(^42\). H–FABP has been associated before with early release following injury of distal renal tubules\(^43\).

The levels below detection limit of this marker we found in the plasma of all animals proved a primary release of urinary H–FABP from kidneys, with minimal release of this protein from heart, skeletal muscle, lungs or brain in this model. **Urine N–acetyl–glucosaminidase (NAG)** release signifies renal damage localized at the level of proximal tubules. Urinary NAG has been measured in renal transplant recipients soon after transplantation to predict acute rejection or chronic allograft nephropathy\(^44\).

In our study, H–FABP and NAG urine concentrations reached significantly higher values as early as half hour and one hour, respectively, after brain death induction, when compared with sham–operated animals. A highly positive correlation was documented between the two renal tubule markers, suggesting a similar pathophysiologic mechanism and consolidating the diagnose of renal tubular damage during brain death. Both proximal and distal renal tubules injuries were early diagnosed in the course of brain death, even before an oxidative hypoxic stress (as shown by \(\text{H}_2\text{O}_2\) generation) took place. The trigger responsible for this early organ injury might be represented by the inflammatory response, shown in our experiments to arise immediately after brain death induction. The values continued to increase progressively during the studied period, which points at an enhanced loss of organ viability with prolongation of the state of brain death.

**In summary**, this study demonstrates immediate pro–inflammatory and pro–coagulatory activation of vascular endothelium after BD in kidney donor rats, proportional with the duration of BD. The mRNA expression of the adhesion molecules E– and P–Selectins, known to promote inflammation by mediating rolling and extravasation of leukocytes, were up–regulated soon (half hour) after brain death induction. Additionally, platelet trapping, most probably due to platelet adhesion to the vascular wall, was visualized as early as half hour after inducing the brain death. To support arguments for an increased thrombogenicity status in brain dead donors, we
report a significant increase in plasma levels of von Willebrand factor, that reflects sustained platelet adhesion to the vascular wall. Additionally we show an increased mRNA expression of \( \alpha \) and \( \beta \) fibrinogen, which will promote extracellular matrix remodelling and thrombogenesis by forming the fibrin network and mediating platelet adhesion. We found for the first time that the brain death related systemic inflammatory response induces an extrahepatic \( \alpha \) and \( \beta \) fibrinogen synthesis. Oxidative stress started to increase after induction of brain death, and became significant only after two hours of BD. Our data point at an ischemic/hypoxic oxidative stress, as main cause of oxygen radicals production, during protracted periods of brain death. BD-related donor kidney damage, reflected by urine concentration of heart-type fatty acid binding protein and N-acetyl-glucosaminidase, was diagnosed as early as half hour in renal tubules, with enhanced loss of viability when the state of brain death was prolonged. These data suggest that early anti-inflammatory and anti-coagulatory therapeutic intervention should be instituted after declaration of brain death, aiming to reduce endothelial activation, prevent platelet adhesion, and possibly slow down an ongoing donor organ deterioration.

Our study also indicates that without effective cytoprotective agents, organ retrieval should not be postponed longer than absolutely necessary, to prevent further injury and loss of viability of scarce donor organs.

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References


