CHAPTER

Slow induction of brain death leads to decreased renal function and increased hepatic apoptosis in rats

D Hoeksma*
RA Rebolledo*
CMV Hottenrott
YS Bodar
PJ Ottens
J Wiersema-Buist
HGD Leuvenink

*Authors contributed equally to the manuscript

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ABSTRACT

Introduction
Donor brain death (BD) is an independent risk factor for graft survival in recipients. While in some patients BD results from a fast increase in intracranial pressure, usually associated with trauma, in others, intracranial pressure increases more slowly. The speed of intracranial pressure increase may be a possible risk factor for renal and hepatic graft dysfunction. This study aims to assess the effect of speed of BD induction on renal and hepatic injury markers.

Methods
BD induction was performed in 64 mechanically ventilated male Fisher rats by inflating a 4.0F Fogarty catheter in the epidural space. Rats were observed for 0.5 h, 1 h, 2 hrs, or 4 hrs following BD induction. Slow induction was achieved by inflating the balloon-catheter at a speed of 0.015 ml/min until confirmation of BD. Fast induction was achieved by inflating the balloon at 0.45 ml/min for 1 minute. Plasma, kidney and liver tissue were collected for analysis.

Results
Slow BD induction led to higher plasma creatinine at all time points compared to fast induction. Furthermore, slow induction led to increased renal mRNA expression of IL-6, and renal MDA values after 4 hrs of BD compared to fast induction. PMN infiltration was not different between fast and slow induction in both renal and hepatic tissue.

Conclusion
Slow induction of BD leads to poorer renal function compared to fast induction. Also, renal inflammatory and oxidative stress markers were increased. Liver function was not affected by speed of BD induction but hepatic inflammatory and apoptosis markers increased significantly due to slow induction compared to fast induction. These results provide initial proof that speed of BD induction influences detrimental renal and hepatic processes which could signify different donor management strategies for patients progressing to BD at different speeds.

INTRODUCTION
The shortage of qualitative donor organs remains a limiting factor in organ transplantation. Therefore, utilization of sub-optimal donor types to meet the increasing demand of organs is inevitable. Today, brain-dead donors form the largest donor pool worldwide for kidney and liver transplantation. Unfortunately, transplanting kidneys from brain dead donors leads to a higher incidence of rejection and delayed graft function compared to living donors. A cerebrovascular cause of BD is related to renal and liver graft failure indicating that the nature of brain insults affect graft function as well. Brain death (BD) is a complex pathological condition, characterized by hemodynamic imbalance, hormonal impairment, and a systemic inflammatory response. Hemodynamic imbalance comprises changes elicited by brainstem herniation, the resulting catecholamine storm, and neurogenic shock due to ischemia of the spinal cord. Systemic inflammation is characterized by increased levels of circulating cytokines including interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β) and, monocyte chemotactic protein 1 (MCP-1). This systemic inflammatory environment promotes the migration of inflammatory cells into organs triggering a local inflammatory and (pro-) apoptotic response. Furthermore, BD affects the pituitary function causing endocrine alterations which are considered to exacerbate the graft deterioration.

Brain death-related processes such as the catecholamine storm are affected by the speed at which intracranial pressure (ICP) increases. A faster increase in ICP leads to higher levels of circulating catecholamines, which is particularly detrimental for cardiac and pulmonary graft function. Indeed, traumatic brain injury, the most common cause of BD preceded by a rapid increase in ICP, is a risk factor for mortality in heart recipients. In contrast, a cerebrovascular cause of death, usually preceded by a slower increase in ICP, is a risk factor for renal and hepatic graft dysfunction. However, this phenomenon is not believed to be associated with a slower increase in ICP. Rather, donor characteristics such as obesity, old age, and the presence of cardiovascular disease are regarded as the underlying cause. We aimed to assess whether the speed of BD induction affects renal and hepatic quality in brain dead donor rats.

MATERIALS AND METHODS
Sixty-four male Fisher F344 rats (270-300 g) were subjected to either fast or slow BD induction with a BD duration of 0.5 h, 1 h, 2 hrs, or 4 hrs. All animals received care in compliance with the guidelines of the local animal ethics committee according to the Experiments on Animals Act (1996) issued by the Ministry of Public Health, Welfare and Sports of the Netherlands. Animals were anaesthetized using 2.5% isoflurane with 100% O2. Two ml of saline 0.9% were administered s.c. to prevent dehydration during surgery. Animals were intubated via a tracheostomy and ventilated (Tidal Volume: 6.5 ml/kg of body weight, PEEP of 3 cm of H2O at an initial respiratory rate of 120 and was adjusted to maintain the ETCO2 in hypocapnic range) throughout the experiment. 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 (Terufusion, Terumo Co., Tokyo, Japan). To prevent movements during catheter inflation, a bolus of rocuronium (0.6 mg/kg) was administered. Fast and slow induction of BD were induced by inflating the catheter at a speed of 0.45 ml/
min or 0.015 ml/min, respectively. For slow speed induction, inflation of the balloon was terminated when the MAP increased above 80 mmHg. For fast induction, the catheter was inflated over a period of one minute. BD was confirmed by the absence of corneal reflexes half an hour after induction after which anesthesia was discontinued. MAP was maintained above 80 mmHg. If necessary, colloid infusion with 10% polyhydroxyethyl starch (HAS) (Fresenius Kabi AG, Bad Homburg, Germany) was given in a bolus (limited to a maximum of 1 ml/h) to maintain the MAP above 80 mmHg. Unresponsiveness to HAS indicated the start of an intravenous noradrenaline (NA) drip (1 mg/ml). A homeothermic blanket control system was used throughout the experiment, maintaining the body temperature between 37 and 38 °C. At the end of the experimental period a bolus of succinylcholine (0.1 mg/kg) was administered in order to prevent movements during aortic puncture, blood and urine were collected. Animals were systemically flushed with cold saline. After the flush, organs were harvested and tissue samples were snap frozen in liquid nitrogen and stored at −80 °C or fixed in 4% paraformaldehyde. Plasma samples and urine were also snap-frozen and stored. One animal was discarded in the slow induction 2 hrs group, two animals in the fast induction group 2 hrs and one in the fast induction 4 hrs group due to unknown amounts of noradrenaline administration. One animal was discarded in the fast induction 4 hrs group due to an apea test conducted during the BD period.

Animals were randomly assigned to one of 8 experimental groups:

- Fast BD induction 0.5 hrs (n = 8)
- Fast BD induction 1 hrs (n = 8)
- Fast BD induction 2 hrs (n = 6)
- Fast BD induction 4 hrs (n = 6)
- Slow BD induction 0.5 hrs (n = 8)
- Slow BD induction 1 hrs (n = 8)
- Slow BD induction 2 hrs (n = 7)
- Slow BD induction 4 hrs (n = 8)

Biochemical determinations

Plasma levels of alanine transaminase (ALT), aspartate transaminase (AST) and creatinine were determined at the clinical chemistry lab of University Medical Centre Groningen according to standard procedures.

Plasma IL-6 measurement

Plasma IL-6 was determined by a rat enzyme-linked immunosorbent assay (IL-6 ELISA) kit (R&D Systems Europe Ltd. Abingdon, Oxon OX14 3NB, UK), according to the manufacturer’s instructions. All samples were analyzed in duplicate and read at 450 nm.

RNA isolation and cDNA synthesis

Total RNA was isolated from whole liver and kidney sections using TRIzol (Life Technologies, Gaithersburg, MD). Samples were verified for absence of genomic DNA contamination by performing RT-PCR reactions in which the addition of reverse transcriptase was omitted, using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers. For cDNA synthesis, 1 μl T7IVN Oligo-dT (0.5 μg/ml) and 1 μg mRNA were incubated for 10 min at 70 °C and cooled directly after that. cDNA was synthesized by adding a mixture containing 0.5 μl RnaseOUT® Ribonuclease inhibitor (Invitrogen, Carlsbad, USA), 0.5 μl RNase water (Promega), 4 μl 5 x first strand buffer (Invitrogen), 2 μl DTT (Invitrogen), 1 μl dNTP’s and 1 μl M-MLV reverse transcriptase (Invitrogen, 200U). The mixture was held at 37 °C for 50 min. Next, reverse transcriptase was inactivated by incubating the mixture for 15 min at 70 °C. Samples were stored at −20 °C.

Real-Time PCR

Fragments of several genes were amplified with the primer sets outlined in Table 1. Pooled cDNA obtained from brain-dead rats was used as an internal reference. Gene expression was normalized with the mean of ß-actin mRNA content. Real-Time PCR was carried out in reaction volumes of 15 μl containing 10 μl SYBR Green mastermix (Applied biosystems, Foster City, USA), 0.4 μl of each primer (50μM), 4.2 μl of nuclease free water and 10 ng of cDNA. All samples were analyzed in triplicate. Thermal cycling was performed on the Taqman Applied Biosystems 7900HT Real Time PCR System with a hot start for 2 min at 50 °C followed by 10 min 95 °C. Second stage was started with 15 s at 95 °C (denaturation step) and 60 s at 60 °C (annealing step and DNA synthesis). The latter stage was repeated 40 times. Stage 3 was included to detect formation of primer dimers (melting curve) and begins with 15 s at 95 °C followed by 60 s at 60 °C and 15 s at 95 °C. Primers were designed with Primer Express software (Applied Biosystems) and primer efficiencies were tested by a standard curve for the primer pair resulting from the amplification of serially diluted cDNA samples (10 ng, 5 ng, 2.5 ng, 1.25 ng and 0.625 ng) obtained from brain-dead rats. PCR efficiency was 1.8 < E < 2.0. Real-time PCR products were checked for product specificity on a 1.5% agarose gel. Results were expressed as 2−ΔΔCT (CT: Threshold Cycle).

<table>
<thead>
<tr>
<th>Table 1: Primers sequences used for Real-Time PCR</th>
<th>Amplitication size (bp)</th>
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<tbody>
<tr>
<td>IL-6</td>
<td>89</td>
</tr>
<tr>
<td>TNF-α</td>
<td>79</td>
</tr>
<tr>
<td>BAX</td>
<td>74</td>
</tr>
<tr>
<td>Bcl2</td>
<td>70</td>
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Tissue Malondialdehyde (MDA)

Kidney and liver tissue were homogenized with a pestle and mortar in PBS containing 5mM butylated hydroxytoluene. MDA was measured fluorescently after binding to thiobarbituric acid. For this, 100μL of tissue homogenate was mixed with 2% SDS followed by 400μL 0.1 N HCl, 50μL 10% phosphotungstic acid and 200μL 0.7% TBA. The mixture was incubated for 30 min at 97°C. To the sample 800μL of 1-butanol were added and centrifuged at 960 g. Of the supernatant 200 μL were used for fluorescence measurements at 480 nm excitation and 590 nm emission wavelengths. Samples were corrected for total amount of protein.

Immunohistochemistry

To detect caspase-3 and HIS48 positive cells in liver and kidney, immunohistochemistry was performed on 3 or 5 μm sections of paraffin embedded samples. Sections were deparaffinized in a sequence of xylene, alcohol and water. As an antigen retrieval method, slides were treated with a 10 mM citrate buffer (pH 6.0) and heated in a microwave oven for 15 min.
we used for caspase-3 samples: EDTA (1mM, pH 8.0) buffer. Next, sections were stained with Caspase-3 primary Antibody (Cell Signaling cat. nr. 9661, 100x diluted in 1% BSA/PBS) using an indirect immunoperoxidase technique. Endogenous peroxidase was blocked using H<sub>2</sub>O<sub>2</sub> 0.3% in phosphate-buffered saline for 30 min. After thorough washing, sections were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG as a secondary antibody for 30 min (Dako, Glostrup, Denmark. cat. nr. P0448), followed by rabbit anti-goat IgG as a tertiary antibody for 30 min (Dako, Glostrup, Denmark. cat. nr. P0449). The reaction was developed using DAB as chromogen and H<sub>2</sub>O<sub>2</sub> as substrate. Sections were counterstained using Mayer hematoxylin solution (Merck, Darmstadt, Germany). For HIS48, mouse monoclonal anti-rat granulocyte antibody (H1S 48; IQ products, Groningen) was dissolved in PBS (pH 7.4) supplemented with 1% bovine serum albumin (BSA). Peroxidase-labeled second antibody (rabbit anti-mouse) was diluted in 1% BSA/PBS containing 5% normal rat serum. Peroxidase activity was visualized using aminoethylcarbazole. Sections were counterstained with Mayers hematoxylin solution. Negative antibody controls were performed. Localization of immunohistochemical staining was assessed by light microscopy. For each tissue section, positive cells per field were counted by a blinded researcher in 10 microscopic fields of the tissue at 10x magnification. Results were presented as number of positive cells per field.

**Statistical Analyses**

Statistical analysis was performed between both experimental groups using a nonparametric test (Mann Whitney) for every time point. Hazard function and the Mantel-Cox were used to compare time of HAES or Noradrenaline administration. All statistical tests were 2-tailed and p < 0.05 was regarded as significant. Results are presented as mean ± SD (standard deviation).

**RESULTS**

As an internal control we compared the catheter volume after brain death induction and blood pressure pattern during the induction phase. The final catheter volume was similar between the slow and fast induction model (0.41 ± 0.03 ml vs 0.41 ± 0.02). During BD induction, the MAP showed different characteristic patterns due to fast and slow speed induction (Figure 1). Slow speed BD induction was characterized by a period of decreased blood pressure which typically started 10 min before the end of the induction and in which the minimum pressure registered was 51.17 ± 10.76 mm Hg. In contrast, fast speed induction was characterized by a sudden and short increase in MAP which was typically observed at the end of the balloon inflation period and in which the maximum pressure registered was 167.39 ± 37.85 mm Hg.

The amount of HAES needed for a stable MAP was similar after fast and slow speed induction. The amount of administered NA was significantly higher in the fast induction group compared to slow induction after 0.5 h and 1 h of BD (Table 2). We estimated the chance of noradrenaline and HAES utilization using hazard curves. Slow induction led to a 17.05% probability of NA use in the first hour of BD, while fast induction led to a 54.84% probability. Additionally, we compared both curves using the Mantel-Cox test. The curves for NA use were significantly different (p = 0.0004). HAES was used mainly in the first minutes after BD induction. Slow induction led to a 48.39% probability of HAES use in the first 10 min of BD while fast induction led to a 84.38% probability. Curve comparison was found to be significantly different using the Mantel-Cox test (p = 0.0091. Figure 2).
ALT and AST plasma levels were measured as liver cell injury markers. No differences were found in ALT levels between fast and slow speed induction. The AST plasma levels were increased due to fast induction compared to slow induction groups after 0.5 and 2 hrs of BD (p = 0.0225 and p = 0.0088, Figure 3).

Plasma creatinine levels were measured in order to estimate kidney function. Creatinine was significantly higher after slow induction compared to fast induction at every time point. Plasma urea levels were increased due to slow induction compared to fast induction after 4 hrs of BD (p = 0.0308, Figure 3).

Plasma IL-6 levels were measured as a marker for systemic inflammation. IL-6 plasma levels were significantly increased due to slow induction compared to fast induction after 0.5 and 1 h of BD (p = 0.0014 and p = 0.0002, Figure 4).
We assessed tissue inflammation by measuring the relative expression of pro-inflammatory genes in the kidney and liver. Relative TNF-α gene expression in the kidney showed no differences between fast and slow speed induction. In contrast, the relative IL-6 gene expression increased significantly due to slow induction compared to fast induction after 0.5 h and 4 hrs (p = 0.0348 and p = 0.0270, Figure 5A). Hepatic TNF-α gene expression increased significantly due to slow induction compared to fast induction after 4 hrs of BD (p = 0.0293). No difference was found in the relative gene expression of IL-6 between fast and slow induction (Figure 5B). PMN infiltration in renal and hepatic tissue was assessed by His-48 staining. There was no difference in His-48 positive staining in the renal cortex and hepatic tissue between fast and slow induction (Figure 6).

Figure 5. Relative expression of inflammatory genes in renal and hepatic tissue in fast and slow induction BD rats after 0.5, 1, 2, and 4 h BD. The fold induction represents the relative expressions of these genes as compared to the expression level of the household GAPDH gene. * indicates p < 0.05.

In order to study apoptotic pathways in renal and hepatic tissue, we measured the ratio between the relative Bax and Bcl-2 expression. No difference was found in the expression of this ratio in renal tissue between fast and slow induction. In contrast, the hepatic gene expression of the Bax/Bcl-2 ratio was significantly higher due to slow induction compared to fast induction after 4 hrs of BD (p = 0.0293, Figure 7). Additionally, hepatic cleaved caspase-3 protein expression was significantly increased due to slow induction compared to fast induction after 4 hrs of BD (p = 0.001, Figure 8).

Figure 6. Polymorphonuclear (PMN) influx in renal and hepatic tissue after 4 h of BD. Kidneys after A) fast induction and B) slow induction. Livers after C) fast induction and D) slow induction.

Oxidative stress was assessed by measuring lipid peroxidation. MDA levels were significantly higher in renal tissue due to slow induction compared to fast induction after 4 hrs of BD (p = 0.01). Hepatic MDA levels were comparable between fast and slow induction groups after 4 hrs of BD (p = 0.48. Figure 9).

Figure 7. Ratio of the relative BAX/Bcl-2 expressions in the kidney and liver in fast- and slow-induced BD rats after 0.5, 1, 2, and 4 h of BD. The fold induction represents the relative expressions of these genes as compared to the expression level of the household GAPDH gene. * indicates p < 0.05.
DISCUSSION

The speed at which brain insults progress to BD varies greatly in ICU patients. Even donors with the same nature of brain insults progress to BD at different speeds. After infarction of the middle cerebral artery, BD can typically manifest itself anywhere between 24 hours and a week. In a prospective study of patients with subarachnoid haemorrhage who progressed to BD, 26% were still not declared BD after one week. This large range in time intervals is the result of the complex pathophysiology of the processes leading to BD and reflective of the speed at which ICP increases. As of yet, the speed at which ICP increases, has not been investigated as a possible determinant of renal and hepatic graft survival.

Here we report for the first time that the speed of BD induction affects functional, immunologic, apoptotic, and oxidative stress markers in the kidney and liver. In our experimental setting, we found that a slower speed of BD induction, elicits more immunologic, apoptotic, and oxidative stress markers in the kidney and liver. In our first time that the speed of BD induction affects functional, as reviewed here and in other studies. While cerebral insults are commonly associated with hypertensive periods, there are a number of reports that associate cerebral insults with hypertensive periods in almost 50% of cases and there is a particularly high risk for iatrogenic induced hypotension. Even a few minutes of hypotension has been associated with an increased risk for acute kidney injury (AKI) possibly aggravated by a dysfunction of the renal blood flow autoregulation. The onset of AKI is reflected by decreased kidney function and increased systemic IL-6 release, as observed here. The extent of IL-6 release can predict mortality in patients and determine the degree of kidney injury. However, systemic IL-6 levels described in this study became comparable between the two BD models at 2 and 4 hrs after BD induction. Therefore, systemic IL-6 levels do not reflect the different local inflammatory responses we observed in our model. Possibly, the combination of AKI and the second hit by BD leads to increased renal IL-6 gene expression which is supported by the increased levels of renal IL-6 gene expression in our model. However, no concurrent increase was observed in PMN infiltration. In non-brain dead animals, others have described an infiltration at 6 hours after AKI onset. However, BD also leads to induction of proinflammatory gene expression and infiltration of immune cells and therefore, possibly no difference was found in PMN infiltration. In AKI, the infiltration correlates with MDA levels in the kidney, mediated by IL-6. In our model, the increased MDA levels in slowly-induced brain-dead rats did not coincide with an increase in PMN infiltration. This indicates that processes other than PMN infiltration affect MDA levels. It was previously shown that renal reactive oxygen species (ROS) start increasing from 2 hours of BD despite hemodynamically stable rats. Therefore, the increase in ROS and lipid peroxidation is likely related to local changes occurring in the kidney. In AKI, oxidative processes mediate peritubular microcirculatory changes which lead to diminished renal perfusion and function. In our model, increased lipid peroxidation could result from the second hit and explain the diminished renal function during the later stages of BD. No difference was observed in hepatic lipid peroxidation between fast and slow BD induction which is in line with AKI as hepatic MDA levels are the result of neutrophil granulocyte infiltration for which we found no difference between models after 4 hours of BD.
In conclusion, the presented data provide an initial broad overview of changes elicited by the speed of BD induction. We found that a slower speed of BD induction leads to more detrimental effects in the kidney and liver. This could indicate that speed of BD induction should be taken into account when decisions about organ allocation are made. The effects of speed of BD induction are more pronounced in the kidney as renal function is diminished more due to a slower speed. Nevertheless, hepatic inflammatory and apoptotic markers are increased more due to slow induction. We believe that increased knowledge about the processes leading up to BD can be of valuable use for brain-dead donor management and thereby improve transplantation outcomes.
REFERENCES


