Inadequate anti-oxidative responses in kidneys of brain-dead rats

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ABSTRACT

Introduction
Brain death (BD)-related lipid peroxidation, measured as serum malondialdehyde (MDA) levels, correlates with delayed graft function (DGF) in renal transplant recipients. How BD affects lipid peroxidation is not known. The extent of BD-induced organ damage is influenced by the speed at which intracranial pressure increases. To determine possible underlying causes of lipid peroxidation, we investigated the renal redox balance by assessing oxidative and anti-oxidative processes in kidneys of brain-dead rats after fast and slow BD induction.

Methods
BD was induced in 64 ventilated male Fisher rats by inflating a 4.0F Fogarty catheter in the epidural space. Fast and slow inductions were achieved by an inflation speed of 0.45 and 0.015 ml/min, respectively, until BD confirmation. Healthy non brain-dead rats served as reference values. Brain-dead rats were monitored for 0.5, 1, 2, or 4 hr(s) after which organs and blood were collected.

Results
Increased MDA levels became evident at 2 hrs of slow BD induction at which increased superoxide levels, decreased GPx activity, decreased glutathione (GSH) levels, increased iNOS and HO-1 expression, and increased plasma creatinine levels were evident. At 4 hrs after slow BD induction, superoxide, MDA, and plasma creatinine levels increased further while GPx activity remained decreased. Increased MDA and plasma creatinine levels also became evident after 4 hrs fast BD induction.

Conclusion
BD leads to increased superoxide production, decreased GPx activity, decreased GSH levels, increased iNOS and HO-1 expression, and increased MDA and plasma creatinine levels. These effects were more pronounced after slow BD induction. Modulation of these processes could lead to decreased incidence of DGF.

INTRODUCTION
Delayed graft function (DGF) is a serious complication in 20-35% of the renal transplant recipients. Kidney grafts from brain-dead donors, the most frequently transplanted grafts, lead to DGF in 15-30% of the cases. These findings cannot be solely attributed to human leukocyte antigen (HLA) mismatches, older donor age, or longer cold ischemia times. Instead, the systemic effects of brain death (BD), which comprise ischemic, inflammatory, and metabolic changes, also affect donor kidney quality and thereby the performance of the future allograft.

Several studies show that BD is associated with oxidative damage of cellular lipid membranes. Lipid peroxidation leads to membrane permeabilization and impairment of enzymatic processes and ion pumps which results in membrane dysfunction and cell toxicity. BD-related lipid peroxidation is correlated with DGF in renal transplant recipients. Levels of malondialdehyde (MDA), a product of lipid peroxidation, in the preservation solution of kidneys retrieved from brain-dead donors correlate well with DGF. Moreover, donor serum MDA levels correlate with acute rejection and immediate and long-term renal allograft function. In expanded criteria donors (ECD), MDA levels in machine perfusion solution also correlate with DGF.

Increased lipid peroxidation can result from increased oxidant production and/or decreased anti-oxidative defenses. Hemodynamic, inflammatory, and metabolic changes can all independently lead to increased oxidant production through enzymes such as xanthine oxidase, NADPH oxidase, nitric oxide synthase, and mitochondrial electron transport complexes. High levels of oxidants or the reaction of oxidants with proteins can lead to the impairment of antioxidant defense systems such as glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD). BD-related processes, such as the catecholamine storm and inflammatory processes are influenced by the speed at which intracranial pressure (ICP) increases. Clinically, traumatic brain insults usually lead to faster increases in ICP and therefore progress to BD more quickly than cerebrovascular causes. Because BD-related processes are influenced by the speed at which ICP increases, it is likely that oxidative and anti-oxidative processes differ between these BD donor types.

Previous reports on oxidative processes in brain-dead donors solely mention the formation of lipid peroxidation products (MDA) in plasma. We hypothesize that the renal redox balance and possible underlying oxidative and anti-oxidative processes are cardinal in the process of lipid peroxidation and that these processes are affected by the speed at which ICP increases. Studying these processes in renal tissue of different BD donor types could provide valuable information for the development of targeted anti-oxidative therapy and thereby improve transplantation outcomes. To assess possible underlying causes of lipid peroxidation, we investigated the renal redox balance by assessing oxidative and anti-oxidative processes in kidneys and plasma of brain-dead rats after fast and slow BD induction.
**CHAPTER 4**

**RENEAL OXIDATIVE STRESS DURING BRAIN DEATH**

**MATERIALS AND METHODS**

**Animal BD model**

The amount of animals per group was calculated using the method of Lenth\(^2\). With a meaningful difference of 50%, a variability (sigma) of 0.3 and a power of 0.8, 9 rats were required per group. Sixty-four male adult Fisher F344 rats (250-300 g) were randomly assigned to either fast or slow BD induction with a BD duration of 0.5, 1, 2, or 4 hrs. These time points were chosen as dynamic effects are observed at these time points which resemble longer clinical brain-dead periods\(^3\). Furthermore, maintaining rats stable for longer periods poses difficulties as cardiac and pulmonary failure become evident. Healthy non-brain-dead rats served as reference values. All animals received care in compliance with the guidelines of the local animal ethics committee according to Experiments on Animals Act (1996) issued by the Ministry of Public Health, Welfare and Sports of the Netherlands.

**BD induction**

Animals were anaesthetized using isoflurane with 100% O\(_2\). Animals were intubated via a tracheostomy and ventilated (tidal volume: 6.5 ml/body weight (Kg) per stroke, PEEP of 3 cm of H\(_2\)O, initial respiratory rate of 120 and corrected based on ETCO\(_2\)) 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 (Terumo, Tokyo, Japan). Fast and slow induction of BD was achieved by infusing the catheter at a speed of 0.015 or 0.45 ml/min, respectively. These speeds were chosen based on consistent results from previous studies\(^1,5\). In the slow induction model, inflation of the balloon was halted once a rise in the MAP above 80 mmHg was noted; reflecting the catecholamine storm at the onset of BD. In the fast induction model the catheter was inflated for 1 minute. BD was confirmed in both groups by the absence of corneal reflexes.

**BD period**

Following confirmation of BD, ventilation was continued and anaesthesia was terminated. Mean arterial pressure (MAP) was considered normal \(\geq 80\) mmHg. If MAP decreased below normal levels, colloid infusion with polyhydroxethyl starch (HAES) 10% (Fresenius Kabi AG, Bad Homburg, Germany) was administered (at a maximum rate of 1ml/hr) to maintain MAP \(\geq 80\) mmHg. If necessary, intravenous noradrenaline (NA) (1 mg/ml) was administered. A homeothermic blanket control system was used throughout the experiment. After the experimental time, blood and urine were collected, after which 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 fixed in 4% paraformaldehyde. Plasma samples were also snap-frozen and stored.

**Determination of superoxide production with dihydroethidium staining**

Four \(\mu\)m cryosections were mounted on slides and washed with Dulbecco’s PBS (DPBS). Sections were incubated with 10 \(\mu\)M dihydroethidium (Sigma, St. Louis, MO) dissolved in DPBS at 37°C in the dark for 30 min. Sections were washed twice with DPBS and immediately scanned for superoxide with a Leica inverted fluorescence microscope equipped with rhodamine filter settings. Images were acquired at 40X magnification and analyzed using NCBI ImageJ.

Anti-oxidative activity assessment of glutathione peroxidase, superoxide dismutase, and catalase

To measure anti-oxidative enzymatic responses to oxidative processes, we measured the activities of glutathione peroxidase, superoxide dismutase, and catalase. Commercially available kit was purchased from Cayman Chemical to perform the assays. The assays were performed according to manufacturer’s protocol and results were expressed as nmol/min/mg protein or as Units/mg protein.

Anti-oxidative activity assessment of glutathione reductase

Glutathione reductase activity measurement was adapted from a method by Griffith\(^2\). Tissue was lysed in cell lysis buffer composed of 253 M HEPES, 5 mM MgCl\(_2\), 5mM EDTA, 2mM PMSF and 10 ng/μl Pepstatine and Leupeptine (Sigma, St. Louis, MO). The buffer was adjusted to a final pH of 7.5. Cell suspensions were centrifuged and the supernatant was analyzed. GSH and GSSG were quantified as follows. Briefly, 20 to 50 μl of the supernatant was added to buffer A (125 mM NaH\(_2\)PO\(_4\), H\(_2\)O and 6.3 mM NaEDTA adjusted to pH 7.5 with NaOH) to a total volume of 100 μl in a transparent flat bottom 96-well plate. Next, 20 μl of 6 mM 5,5’-dithiobis-2-nitrobenenate (Sigma, St. Louis, MO), 42 μl of 0.3 mM NADPH (Roche Diagnostics, Germany), and 10 μl of 1mM GSH solution, all dissolved in buffer A, were added to each well. The final volume of the mixture was increased to 200 μl by adding buffer A. The absorbance was measured at 430 nm for 15 minutes at 30 °C. The linear part of the kinetic curve was used for the rate estimation and compared with a standard curve of glutathione reductase (GR). Samples were corrected for total amount of protein and expressed as Units/mg protein.

**Intracellular redox status assessment by reduced (GSH) and oxidized glutathione GSSG measurements**

Reduced and oxidized glutathione were measured according to the method of Griffith\(^2\). Tissue was lysed in cell lysis buffer composed of 253 M HEPES, 5 mM MgCl\(_2\), 5mM EDTA, 2mM PMSF and 10 ng/μl pepstatin and leupeptin (Sigma, St. Louis, MO). The buffer was adjusted to a final pH of 7.5. Cell suspensions were centrifuged and the supernatant was analyzed. To measure total glutathione, 20 μl of the supernatant or plasma was added to buffer A (125 mM NaH\(_2\)PO\(_4\), H\(_2\)O and 6.3 mM NaEDTA adjusted to pH 7.5 with NaOH) to a total volume of 100 μl in a transparent flat bottom 96-well plate. Next, 20 μl of 6 mM 5,5’-dithiobis-2-nitrobenenate (Sigma, St. Louis, MO), 42 μl of 0.3 mM NADPH (Roche Diagnostics, Germany), all dissolved in buffer A, were added to the wells. Finally, 38 μl of glutathione reductase (Roche Diagnostics, Germany) dissolved to an enzyme activity of 5 units/ml in Buffer A was added to the wells. The absorbance was measured at 430 nm for 15 minutes at 30 °C. The linear part of the kinetic curve was used for the rate estimation and compared with a standard curve of GSSG. Samples were corrected for total amount of protein and expressed μmol/g protein. In order to measure GSSG, 1-methyl-2-vinyl pyridinium triolate (Sigma, St. Louis, MO) was added at a concentration of 3 mM to the supernatant to block GSH. GSH content was calculated by subtracting GSSG from the total glutathione values whilst correcting for the molecular weight of the molecules.

**RNA isolation and qPCR**

RNA isolation and qPCR were performed as described before\(^4\) In brief, the SV Total RNA isolation kit was used to isolate total RNA from rat kidneys. Genomic DNA contamination was verified by RT-PCR reactions by omitting...
Due to a prolonged apnea test because of uncertainty of BD.

One animal was discarded in the fast induction 4 hrs group and one from the fast induction 4 hrs group were discarded due to unknown amounts of noradrenaline administration. One animal from the slow induction 2 hrs group, 2 from the fast induction 2 hrs group and slow induction groups were compared to reference groups using the Kruskall-Wallis test with Dunns post-hoc correction.

RESULTS

Hemodynamic support

More noradrenaline administration was required after fast compared to slow BD induction at 0.5 and 1 hrs (Table 2) which was not observed at other time points. No differences in HAES administration were observed between fast and slow BD induction at the different time points.

<table>
<thead>
<tr>
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<th>Fast Induction</th>
<th>Slow Induction</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.35 ± 0.42 (7)</td>
<td>0.10 ± 0.24 (2)</td>
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<td>0.55 ± 0.76 (6)</td>
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<td>0.0238*</td>
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<tr>
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<td>1.1 ± 1.6 (4)</td>
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<td>0.1515</td>
</tr>
<tr>
<td>4</td>
<td>0.33 ± 0.58 (2)</td>
<td>0.23 ± 0.42 (2)</td>
<td>0.8564</td>
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</table>

* indicates a significant difference between fast and slow induction groups.

Plasma creatinine values

Plasma creatinine values were significantly increased after 4 hrs by fast induction compared to reference values (p < 0.01). After slow induction, creatinine values were significantly increased at 0.5, 1, 2, and 4 hrs compared to reference values (p < 0.05, 0.05, 0.01 and 0.001 respectively. Table 3). Plasma creatinine values were significantly increased by slow compared to fast induction BD at 0.5 hrs (p < 0.05).

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* indicates a significant difference between fast and slow induction groups.

Superoxide production with dihydroethidium staining

Superoxide was increased at 0.5 hrs of BD by both fast- and slow BD induction compared to reference values (p < 0.001 and p < 0.05, respectively, Figure 1). Marked increases in superoxide production were also observed at 2 and 4 hrs after slow induction compared to reference values (p < 0.01, and p < 0.001). Superoxide was significantly increased after slow BD induction at 4 hrs compared to fast induction (p < 0.05).

RENEWAL OXIDATIVE STRESS DURING BRAIN DEATH

Plasma lactate levels were significantly increased at 4 hrs by fast induction compared to reference values (p < 0.01). After slow induction, lactate levels were significantly increased at 0.5, 1, 2, and 4 hrs compared to reference values (p < 0.05, 0.05, 0.01 and 0.001 respectively. Table 3). Plasma lactate values were significantly increased by slow compared to fast induction BD at 0.5 hrs (p < 0.05).

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Plasma lactate levels were significantly increased at 4 hrs by fast induction compared to reference values (p < 0.01). After slow induction, lactate levels were significantly increased at 0.5, 1, 2, and 4 hrs compared to reference values (p < 0.05, 0.05, 0.01 and 0.001 respectively. Table 3). Plasma lactate values were significantly increased by slow compared to fast induction BD at 0.5 hrs (p < 0.05).

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Glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was significantly decreased after slow induction of BD at 0.5, 1, and 2 hrs compared to reference values (p < 0.05, 0.01, and 0.05, respectively, Figure 3) but increased at 4 hrs. After slow induction of BD, GPx activity decreased significantly at 1 hr and remained decreased at 2 and 4 hrs compared to reference values (p < 0.05, 0.05, and 0.01, respectively, Figure 3).

SOD activity

SOD activity remained stable at different BD time points. No differences were observed between groups at different time points (Figure 3).

Catalase activity

Catalase activity was not affected by either fast or slow induction compared to reference values. No differences were observed between groups at different time points (Figure 3).

Glutathione reductase activity

Glutathione reductase activity decreased significantly after fast BD induction at 0.5 hrs compared to reference values (p < 0.05, Figure 3). Furthermore, the activity was significantly increased after slow BD induction at 4 hrs compared to fast induction (p < 0.01, Figure 3).

GSH, GSSG, GSH + GSSG (total glutathione) levels and GSSG:GSH ratio

GSH levels decreased after both fast- and slow BD induction but only reached significance after slow induction at 2 hrs compared to reference values (p < 0.05). GSSG levels were significantly increased after slow induction of BD compared to reference values (p < 0.01, Figure 4). Furthermore, GSSG levels were significantly increased after slow BD induction at 4 hrs compared to fast induction (p < 0.05). Total glutathione levels were unchanged after both fast and slow BD induction. The GSSG:GSH ratio did not change significantly between groups at different time points.
Renal gene expression levels

iNOS and HO-1 gene expression were increased at all BD time points after both fast- and slow BD induction. Fast induction resulted in significantly increased iNOS gene expression compared to reference values after 2 and 4 hrs of BD (p < 0.01 and 0.001, respectively, Figure 6). Slow induction resulted in earlier increases, namely after 1, 2, and 4 hours of BD (p < 0.01, 0.01, and 0.001, respectively, Figure 6). HO-1 gene expression showed a similar pattern in that slow induction of BD led to an earlier increase. HO-1 gene expression was significantly increased after fast induction of BD at 2 and 4 hours compared to reference values (p < 0.05 and 0.001, respectively, Figure 6) while after slow induction of BD, significantly increased HO-1 gene expression was observed at 1, 2, and 4 hrs compared to reference values (p < 0.05, 0.01, and 0.001, respectively, Figure 6). Finally, HO-1 gene expression was significantly higher after slow BD induction at 4 hours compared to fast induction (p < 0.05).
The main finding of this study is that BD leads to increased renal superoxide production, decreased GPx activity, decreased GSH levels, increased iNOS and HO-1 expression, increased MDA levels, and increased plasma creatinine levels. The effects we observed were more pronounced when BD was induced slowly which might explain differences in performance of donor kidneys derived from different donor types. The fact that graft quality is affected by the speed at which ICP increases has been shown previously. A faster increase in ICP leads to increased myocardial damage and decreased function which is likely related to higher levels of hypertension due to increased catecholamine release. Traumatic brain injury, which usually leads to a quick rise in ICP, is a risk factor for mortality in heart recipients. In contrast, our study shows that a slower increase in ICP leads to increased lipid peroxidation. Rather than hypertension, this could be related to hypotension and the resulting ischemia, which is observed in patients with cerebrovascular brain insults. These patients tend to progress to BD less quickly than patients who suffer traumatic brain injury. Cerebrovascular causes of death in the donor are indeed a risk factor for renal graft dysfunction in transplant recipients. However, this increased risk is attributed to donor specific characteristics such as age, hypertension, and cardiovascular disease, while we show here that the speed at which ICP increases could be of influence as well. Regardless of the nature of brain insults, the speeds at which brain injuries lead to BD vary greatly between patients. In a series of patients with middle cerebral artery infarction, BD occurred anywhere between 24 hours and one week. A similar time range was evident in patients with subarachnoid hemorrhage. Therefore, anti-oxidative therapy could be especially beneficial in donors who progress to BD slowly after cerebral insults, regardless of the nature of the insult.

BD leads to phases of renal ischemia as a result of the catecholamine storm, volume depletion, and neurogenic shock. In classic models of renal ischemia-reperfusion (I-R) injury, increased superoxide production, decreased GPx, SOD, and CAT activity, increased GSH levels, and increased MDA and plasma creatinine levels are observed. Similar effects were observed in our BD model. In many models, increased superoxide production takes a central role in decreasing GPx activity, GSH levels, MDA levels, and increasing plasma creatinine levels. Considering the time points at which superoxide production is increased in our model, it is likely that superoxide also fulfills a central role in initiating the effects observed in our model, including lipid peroxidation, and therefore increases the risk of DGF in transplant recipients. Thus, the administration of superoxide scavengers to brain-dead donors, especially those progressing to BD slowly, could lead to improved renal transplantation outcomes. Furthermore, considering the decreased GPx activity at almost all time points and its specific role in protecting lipid membranes, the administration of GPx mimetics could exert beneficial effects as well. The early effects we observed at 0.5 hours are likely the result of major hemodynamic changes such as the catecholamine storm which is specific for the onset of BD. The effects observed at later stages are likely not related to major hemodynamic disturbances as rats are kept hemodynamically stable. Therefore, changes in the local renal circulation probably affect oxidative processes and renal function at later time points. In acute kidney injury (AKI) models, local renal circulatory changes become apparent hours after renal ischemia. In BD, early hemodynamic changes could represent the first ischemic hit after which local renal circulatory changes take place at later time points. Since the onset of BD cannot be anticipated precisely, counteracting early hemodynamic changes could be difficult. However, the later oxidative processes, which probably do not involve hemodynamic dermaliments, could however effectively be counteracted with anti-oxidative regimens as explained above.

In our BD model, the GSSG:GSH ratio was unaltered at all time points which indicates that glutathione homeostasis is not affected overall. Augmenting glutathione levels in brain-dead donors is therefore most likely futile. In a recent randomized controlled trial, n-acetylcysteine, a glutathione precursor, was administered to deceased donors but no effect was found on renal graft function or survival in recipients. Like in I-R models, glutathione levels decreased in our model but were unaltered at 4 hours despite the oxidative state at the time. This finding is probably not solely explained by increased glutathione reductase activity since GSSG levels are significantly increased at the time.
Renal glutathione synthesis is totally dependent on renal uptake of glutathione conjugates from the circulation\(^{42,43}\). We observed increased total glutathione plasma levels after slow induction of BD which could be the result of BD-related hepatic apoptosis which is significantly higher after slow compared to fast BD induction\(^{44}\).

Besides detrimental effects, BD also led to the compensatory upregulation of cytoprotective genes\(^{31}\). We observed marked increases in renal iNOS and HO-1 expression. iNOS can exert a protective effect through suppression of inflammatory reactions and increasing blood flow to ischemic regions\(^{44,45}\). However, higher levels of iNOS expression after slow induction did not coincide with decreased lipid peroxidation levels. Furthermore, increased NO production in an oxidative environment probably exerts a negative effect as NO will quickly react with oxidants such as superoxide, resulting in the production of peroxynitrite, a highly reactive oxidant\(^{46}\). Therefore, increasing NO production in brain-dead donors will probably not exert beneficial effects. Heme-oxygenase 1 (HO-1) is among the most critical cytoprotective enzymes that are activated upon cellular stress\(^{47}\). The increased renal expression of HO-1 in BD is therefore believed to form part of a recuperative mechanism\(^{36}\). However, in our model, increased HO-1 gene expression did not coincide with decreased lipid peroxidation after slow induction of BD. Probably, the induction of HO-1 by BD is either insufficient or occurs too late to prevent lipid peroxidation. The induction of HO-1 prior to BD has however proven beneficial in allograft survival in a rat transplantation model\(^{42,43}\). Therefore, inducing HO-1 in brain-dead donors could pose clinical difficulties as HO-1 might already need to be induced prior to BD to exert beneficial effects.

In conclusion, this study shows oxidative and anti-oxidative effects elicited by fast and slow BD induction. The observed effects could form an explanation for the increased lipid peroxidation observed in brain-dead donors. Since BD-related lipid peroxidation correlates with DGF in renal transplant recipients, anti-oxidative therapy in brain-dead donors could decrease lipid peroxidation and thereby improve transplantation outcomes.

**REFERENCES**

CHAPTER 4

RENEWED INTEREST IN \textit{Malignant} brain death


