CHAPTER 8

Donor pretreatment with carbamylated EPO in a brain death model reduces inflammation more effectively than EPO while preserving renal function

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Published in Critical Care Medicine 2010 Jan 29.
Abstract

Objective:
We hypothesized that donor treatment of deceased brain dead donors would lead to a decrease in inflammatory responses seen in brain death and to a restoration of kidney function.

Design:
A standardized slow induction rat brain death model followed by evaluation of kidney function in an isolated perfused kidney model.

Settings:
Surgery Research Laboratory, University Medical Center Groningen.

Subjects: Male Fisher rats.

Interventions:
Donor treatment with erythropoietin (EPO), carbamylated EPO (cEPO) which lacks erythropoietic activity, or vehicle.

Measurements and Main Results:
In brain death, cEPO, and to a lesser extent EPO, was able to decrease the expression of several proinflammatory genes and to decrease the infiltration of PMNs in the kidney. No effect on tubular injury parameters was seen. Kidney function decreased almost by 50% after brain death, but was fully restored after treatment with both cEPO and EPO.

Conclusions:
cEPO can inhibit the inflammatory response caused by brain death more effectively than EPO, while both substances can restore kidney function after brain death.
- Introduction -

For most patients with end-stage renal failure kidney transplantation is the therapy of choice. The majority of donor organs is retrieved from heart beating deceased brain dead (DBD) donors, although donor shortage has lead to an increased use of kidneys from living donors (LD) or from donors after cardiac death (DCD). Living donor grafts are associated with better survival and lower rates of delayed graft function than kidneys retrieved from DBD donors (1;2). Also, cerebral injury and brain death in the donor may be a risk factor for developing vascular rejection after transplantation (3).

This difference in success between LD and DBD donors can not be fully explained by factors such as warm and cold ischemia times or HLA mismatches (1). The difference can at least be partly attributed to pathophysiological changes in the donor which take place during the brain death process (4;5). Experimental as well as clinical studies have shown that brain death negatively affects hemodynamic stability and hormone regulation in the donor (6;7). Recently, our group as well as others have demonstrated that brain death induces a marked endothelial activation and substantial pro-inflammatory responses in potential donor organs, which may enhance the immunogenicity of the graft-to-be thereby affecting the allograft response in the recipient (8-10). Pretreatment of the DBD donor to counteract these effects may therefore be an elegant option to improve transplantation outcome.

Erythropoetin (EPO) is a pleiotropic cytokine originally identified for its role in erythropoiesis (11). In the last years, it has become clear that EPO also exerts a number of cytoprotective functions. Treatment with EPO was found to be neuroprotective after ischemia, by diminishing apoptosis and necrosis in the brain (12). Also, EPO stimulates cardiomyocyte proliferation and decreases myocardial infarct size (13;14). In the kidney, administration of EPO leads to heat shock protein production, and decreases apoptosis and serum creatinine in ischemia/reperfusion (15). Furthermore, EPO helps to preserve endothelial integrity and to diminish inflammation (16-18). These effects make EPO an interesting candidate for improving graft quality in DBD donors. However, despite its promise, EPO treatment is also associated with some (detrimental) side effects: increasing the hematocrit, an elevation of blood pressure and increased risk of thrombosis (19).

These disadvantages might be neutralized by using a modified EPO derivative. Recently, several studies have been published regarding the use of carbamylated EPO (cEPO). While EPO signals through the homodimeric erythropoietin receptor (EPOR) to exert its hematopoietic effects, cEPO acts upon a heteroreceptor complex comprising both EPOR and the common β receptor subunit (βcR, also known as CD131). It has been shown that cEPO retains its tissue protective effects but does not have any hematopoietic activity (20;21). Recently, several studies have been published regarding the use of cEPO. It was shown that cEPO protects against radiation-induced brain injury and ischemia/reperfusion injury in the myocardium (22;23). In a comparison of cEPO and EPO treatment in an ischemia/reperfusion model of the kidney, cEPO was shown to be more successful in inhibiting apoptosis and stimulating proliferation in tubular cells (24).
In this study we have investigated the cytoprotective effects from both EPO and cEPO on the endothelium and its anti-inflammatory properties. We postulated that both forms of erythropoietin would exert protective effects on the kidney graft-to-be during brain death. Therefore, we have investigated the effects of both EPO and cEPO in a rat brain death model. In addition, we have tested the effect of both substances on kidney function after donation.

- Materials and Methods -

**Animals**
Adult male Fisher F344 rats (250-300 g) were used. 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 Netherlands Ministry of Public Health, Welfare and Sports.

**Experimental groups**
To study the effects of erythropoietin (EPO), as well as the effects of carbamylated erythropoietin (cEPO) administration in the DBD donor, four groups (n=6) were studied.

- Group 1: Administration of cEPO 10 μg/kg i.v., 4h brain death, kidney reperfusion in isolated perfused kidney model (IPK)
- Group 2: Administration of EPO 10 μg /kg i.v., 4h brain death, kidney reperfusion in IPK model
- Group 3: Administration of vehicle i.v., 4h brain death, kidney reperfusion in IPK model
- Group 4: Sham operated control group, kidney reperfusion in IPK model

EPO and cEPO were provided by Warren Pharmaceuticals, Ossining, NY.

**Brain death model**
Brain death was induced as described previously (25). Briefly, the procedure was as follows: Animals were anesthetised using isoflurane with O2. A cannula was inserted in the femoral artery for blood pressure monitoring. Animals were intubated via a tracheostomy and ventilated throughout the experiment. Through a frontolateral borehole in the skull a no. 4 Fogarty catheter (Edwards Lifesciences Co., Irvine, CA) was placed subdurally and slowly inflated (16 μL/min) with saline using a syringe pump (Terufusion, Termo Co., Tokyo, Japan). Inflation of the balloon was stopped during the subsequent sharp rise in blood pressure, which reflects the autonomic storm at the beginning of brain death. This typically occurred 30min after starting balloon inflation. Brain death was confirmed by the absence of corneal and pupillary reflexes and an apnea test. During brain death, animals received no anesthesia. If blood pressure fell below 80 mmHg, it was restored by the administration of HAES 10%
(Fresenius Kabi AG, Bad Homburg, Germany). Temperature was monitored rectally and kept constant. Animals were kept brain dead for 4h.

At the end of the brain death period, donors were heparinized with 500 IU heparin. A laparotomy was performed and blood and urine were collected from the aorta and the bladder. Next, kidneys were flushed with saline and removed. The left kidney artery was cannulated, weighed and placed in an isolated perfused kidney setup (IPK) for evaluation of kidney function. Blood and urine were centrifuged for 10 min. at 960g. Urine supernatant and blood plasma were collected and stored at -80°C. Tissue from the right kidney was snap frozen in liquid nitrogen. Sham operated rats, which had been anesthetized for the sham operation (all procedures except intracranial balloon inflation) and for 30 min thereafter to mimic the induction of brain death served as controls.

Reperfusion with the Isolated perfused kidney model
The left kidney was evaluated in the isolated perfused kidney (IPK) model as described before (26-28). In this model, the kidney artery is cannulated, placed in an organ chamber and perfused using a warmed and oxygenated perfusion fluid supplemented with creatinine. In this way, renal function can be measured without concomitant factors due to a recipient in a transplantation model. A draw back of the model is that fractional sodium reabsorption is less than in vivo. The kidney was continuously perfused via the renal artery with warmed (37°C) and oxygenated (95% O₂ and 5% CO₂ gas mixture) Krebs-Henseleit-bicarbonate (KHB) solution with added amino acids at a pH of 7.4 ± 0.05, PO₂ > 100 kPa, by using a roller pump (Ismatec mv-ca/04, Ismatec, Glattbrugg, Switzerland) delivering a constant flow throughout the experiment. Creatinine 0.8 g/L was added to the perfusion solution to be able to measure creatinine clearance. After connecting the kidney, vascular responses were monitored by an electromechanical pressure transducer (Cobe, Arvada, USA) and the flow was gradually increased in the first 10 minutes to reach a kidney perfusion pressure of 100 mmHg. Kidneys were perfused during 90 min. Samples of urine and perfusate were were collected at 15-min intervals throughout the experimental period and stored at -80°C. Volumes of urine production were recorded during the experiment. A scheme of the experimental setup is shown in Figure 1.
**Biochemical determinations**

At the Laboratory Center of the University Medical Center Groningen (Mega, Merck), the following measurements were determined in a routine fashion: Creatinine in plasma, perfusate and urine (by the Jaffé method (29)), alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) enzyme activity in plasma (30), and lactate dehydrogenase (LDH) activity in plasma (based on the conversion rate of lactate to pyruvate). Sodium in urine and perfusate was determined by flame photometry.

Glomerular filtration rate was approximated by creatinine clearance rate during the Isolated Perfused Kidney setup which was calculated using the formula:

\[
\text{Creatinine clearance} = \frac{[\text{creatinine}]_{\text{urine}} \times \text{urine volume}}{[\text{creatinine}]_{\text{perfusion fluid}}}
\]

Fractional sodium excretion was calculated during the Isolated Perfused Kidney setup using the formula:

\[
\text{Sodium excretion \%} = \left( \frac{[Na]_{\text{urine}} \times [\text{creatinine}]_{\text{perfusion fluid}}}{[Na]_{\text{perfusion fluid}} \times [\text{creatinine}]_{\text{urine}}} \right) \times 100.
\]

Activity of the brush border enzyme alanine aminopeptidase (AAP) was measured in urine to determine the amount of tubular damage. The method used is based on hydrolysis of L-alanine-p-nitroanilide into p-nitroaniline. Enzyme activity was expressed as the amount of enzyme required to release 1 μmol of product per minute.
Malondialdehyde was estimated by measuring the amount of thiobarbituric acid-reactive substances (TBARS) in plasma. TBARS are formed when free oxygen radical molecules react with proteins and phospholipids in membranes and is therefore a measure of lipid peroxidation and oxidative damage. TBARS levels were determined by a method based on the reaction with thiobarbituric acid (TBA) at 92–97°C, producing a pink pigment having an absorption maximum at 530 nm.

**Real-Time RT-PCR on renal tissue**

To investigate differences in gene expression, total RNA was isolated from snap frozen cross-section kidney slices, using the TRIzol method and including a DNase treatment step. RNA samples were verified for absence of genomic DNA contamination by performing RT-PCR reactions, in which the addition of reverse transcriptase was omitted, using GAPDH primers. Gene-specific primers were designed using the computer program Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA) using published gene sequences. Studied genes, primer sequences and product sizes are given in Table 1.

**Table 1  Primer sequences used**

<table>
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<th>gene</th>
<th>primer sequences</th>
<th>accession number</th>
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<td>β-actin</td>
<td>GGAAATCGTGCGTGACATTAAA</td>
<td>NM_031144.2</td>
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<td></td>
<td>GCGGCAGTGGGGACTCTCT</td>
<td></td>
<td></td>
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<td>Epo</td>
<td>TCACGAAGCCATAGAACAGACAGA</td>
<td>NM_017001.1</td>
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<td></td>
<td>GCTGTTCGCCAGTGAGATTTTTA</td>
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<td></td>
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<td>Epo-receptor</td>
<td>TTGGTGTGTCTCGGGAGGA</td>
<td>NM_017002.2</td>
<td>67</td>
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<td></td>
<td>AGCTGGTAAGAGAAGGCTGATGGTGA</td>
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<td>TNF-α</td>
<td>AGGTCTGCGCTACACTGAA</td>
<td>NM_012675.2</td>
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<td></td>
<td>TGACCCGTAGGGGAGTATTACA</td>
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<tr>
<td>IL-1β</td>
<td>CAGCAATGCTCGGACATAGTT</td>
<td>NM_031512.1</td>
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<td></td>
<td>GCTATGGAAATAGTGCGACATCTCT</td>
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<td>IL-6</td>
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<td>TCAAGTCTCTTCAAGATGGTGGAT</td>
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<td>e-selectin</td>
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<td>CTGCCACAGAAGTGGCACTAC</td>
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<tr>
<td>p-selectin</td>
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<td>NM_013114.1</td>
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<td>GTGGCCCTACTATCCATCTGGA</td>
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<td>VCAM-1</td>
<td>TGTGGAAATGCGCCGAAA</td>
<td>NM_012889.1</td>
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<tr>
<td></td>
<td>ACAGGCCATTTACAGACTTTTACGA</td>
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<td>Kim-1</td>
<td>AGAGAGAGCGAGACACAGGCTT</td>
<td>NM_173149.1</td>
<td>75</td>
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<td></td>
<td>ACCCGTGGTATCTCCAAACA</td>
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<tr>
<td>BAX</td>
<td>GCGTGGTGGCTCCTCTCTAC</td>
<td>NM_017059.1</td>
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<td>TGATCAGCTCGGGCATTGTAG</td>
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<tr>
<td>BCL-2</td>
<td>CTGCGATGCCCTTGGGAGAA</td>
<td>NM_016993.1</td>
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<tr>
<td></td>
<td>TCAAGAGACAGCGAGAAATCAA</td>
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<td>PAI-1</td>
<td>GCACAGGAGGTACAGTGAATCTA</td>
<td>NM_012620.1</td>
<td>72</td>
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<td></td>
<td>TTTTCTTCCAGTGGAGATGTAAACG</td>
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<td>HO-1</td>
<td>CTCGAGATGAAGAACCTCGGGAGAT</td>
<td>NM_012580.2</td>
<td>74</td>
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<td></td>
<td>GCAGGAAGGGGCTCTTACGC</td>
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</table>
Amplification and detection of PCR products were performed with the Applied Biosystems 7900 HT Real Time PCR System (Applied Biosystems, Foster City, USA) using emission from SYBR Green (SYBR Green master mix, Applied Biosystems). All assays were performed in triplicate. After an initial activation step at 50 ºC for 2 min. and a hot start at 95ºC for 10 min., PCR cycles consisted of 40 cycles at 95ºC for 15 sec. and 60ºC for 60 sec. Specificity of qPCR products was routinely assessed by performing a dissociation curve at the end of the amplification program. Gene expression was normalized using the mean of β-actin gene expression from the same sample and calculated relative to controls using the relative standard curve method. Results were finally expressed as $2^{\Delta \Delta CT}$ (CT threshold cycle).

**HIS-48 staining on renal tissue cryosections**

To detect polymorphonuclear cells (PMNs) in the kidney, immunohistochemistry was performed on 5 μm kidney tissue cryosections. Sections were fixated for 10 minutes using acetone. Next, sections were stained with HIS-48 mAb (supernatant, two times diluted) using an indirect immunoperoxidase technique. Endogenous peroxidase was blocked using H$_2$O$_2$ 0.01% in PBS for 30 minutes. After thorough washing, sections were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG as a secondary antibody for 30 min., followed by goat anti-rabbit IgG as a tertiary antibody for 30 min. (both from Dako, Glostrup, Denmark). The reaction was developed using 9-amino-ethylcarbazole as chromogen and H$_2$O$_2$ as substrate. Sections were counterstained using Mayer’s hematoxylin solution (Merck, Darmstadt, Germany). Negative antibody controls were performed. Localization of immunohistochemical staining was assessed by light microscopy. For each tissue section, positive cells were counted in 10 microscopic fields of the cortex at 40x magnification.

**Statistical Methods**

Statistical analysis was performed using the computer program SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). Groups were tested for normal distribution using the One-Sample Kolmogorov-Smirnov Test. Results are expressed as arithmetic means ± standard error of the mean. To investigate differences in kidney function during IPK, AUCs (area under the curve) for creatinine clearance were calculated. Statistical comparisons between unpaired groups were performed using ANOVA. All differences were considered to be significant at P<0.05.
- Results -

**Brain death experiments**

Induction of brain death showed a consistent and uniform pattern in blood pressure as described before (25) and took approximately 30 minutes. All animals were kept at a mean arterial pressure of at least 80 mmHg during the experiment. In the brain dead groups, infusion of 2.39 mL ± 0.42 of HAES 10% was needed to maintain stable blood pressures. No difference in required HAES infusion between brain dead groups was found (P=0.41).

**Biochemistry after brain death**

Blood was collected at the end of the animal experiments. After brain death, creatinine levels in plasma were 116 μmol/L ± 9.7, compared to 72 ± 6.5 in sham operated animals (P=0.002). EPO and cEPO administration had no effect on creatinine levels. In urine, no significant differences in creatinine levels were found between brain dead compared with sham operated animals (9.0 ± 1.2 mmol/L vs 9.8 ± 1.5 mmol/L, P=0.36). Both EPO and cEPO administration diminished urinary creatinine concentration (EPO: 4.5 ± 0.25 mmol/L, cEPO: 5.3 ± 0.26 mmol/L). Calculation of creatinine clearance during brain death was not possible, as the total urine production could not be measured reliably during the brain dead period.

To evaluate cellular damage, urine alanine aminopeptidase (AAP), and plasma lactate dehydrogenase (LDH), alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), thiobarbituric acid-reactive substances (TBARS) were measured. LDH was increased significantly by brain death, but cEPO administration decreased LDH release to levels seen in the sham operated group. On the other damage markers, no effect from EPO or cEPO administration was seen.

**Table 2  Cellular injury markers in urine and plasma**

<table>
<thead>
<tr>
<th></th>
<th>sham</th>
<th>BD</th>
<th>BD + cEPO</th>
<th>BD + EPO</th>
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</thead>
<tbody>
<tr>
<td>AAP (mM/min)</td>
<td>0.7 ± 0.1</td>
<td>11.1 ± 2.0*</td>
<td>14.9 ± 2.5*</td>
<td>13.9 ± 1.7*</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>150 ± 21</td>
<td>226 ± 49</td>
<td>148 ± 16†</td>
<td>309 ± 43*</td>
</tr>
<tr>
<td>ALAT (U/L)</td>
<td>48 ± 3</td>
<td>54 ± 7</td>
<td>52 ± 5</td>
<td>47 ±6</td>
</tr>
<tr>
<td>ASAT (U/L)</td>
<td>60 ± 4</td>
<td>88 ± 7</td>
<td>81 ± 10</td>
<td>80 ± 9</td>
</tr>
<tr>
<td>TBARS (μM)</td>
<td>2.1 ± 0.1</td>
<td>2.4 ± 0.1</td>
<td>2.4 ± 0.4</td>
<td>2.6 ± 0.4</td>
</tr>
</tbody>
</table>

AAP: alanine aminopeptidase; LDH: lactate dehydrogenase; ALAT: alanine aminotransferase; ASAT: aspartate aminotranspherase; TBARS: thiobarbituric acid-reactive substances

* significant at p<0.05 as compared to sham operated controls
† significant at p<0.05 as compared to BD
Gene expression in the kidney

Outcomes of RT-PCR on kidney tissue collected after the animal experiments are summarized in Figure 2 and Table 3.

As EPO administration can downregulate EPO production (31), gene expression of EPO and EPO receptor (EPOr) was determined in the kidney. EPO but not cEPO administration decreased EPO production significantly (mean fold induction 0.2 vs 1.1 in sham operated animals, P=0.004, Fig 2).

Inflammation and endothelial cell activation were studied by measuring gene expression of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6, and the adhesion molecules e-selectin, p-selectin and vascular cellular adhesion molecule-1 (VCAM-1).

For IL-6 a profound 85-fold induction of gene expression was seen after brain death compared to sham operated animals (P=0.002), while cEPO treatment reduced this to a 25-fold induction (P=0.003). EPO treatment did not have any beneficial effect (Fig 2). Treatment with cEPO reduced IL-1β expression from na 8.4-fold to a 2.6-fold induction (p=0.01 compared to BD without treatment), whereas EPO treatment did not have this effect (Fig 2). For e-selectin gene expression, an 9.2-fold induction was found after brain death, compared with sham operated animals. This was only decreased significantly by cEPO administration. VCAM-1 gene expression was slightly but significantly downregulated after both EPO and cEPO administration.

Figure 2 Kidney gene expression
Kidney injury molecule (Kim-1) was studied to assess tubular damage. A 83-fold Kim-1 gene induction was found after brain death, compared with sham operated animals (P=0.004). EPO and cEPO treatment had no effect on Kim-1 gene expression (Fig 2).

Apoptosis was studied by calculating BAX/BCL-2 ratios, where an increased ratio signifies an increased level of apoptosis. cEPO and EPO did not have any effect on this ratio.

As EPO might exert some of its cytoprotective effects during hypoxia through stimulation of the cytoprotective heme oxygenase-1 (HO-1) (32), HO-1 gene expression was determined. Finally, PAI-1 gene expression was determined, as even a short time of exposure to EPO is known to increase the production of plasminogen activator inhibitor-1 (PAI-1) in endothelial cells (35). In this study, EPO and cEPO administration had no effects on HO-1 and PAI-1 gene expression.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Kidney gene expression</th>
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<tr>
<td>Fold induction</td>
<td>BD</td>
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<tr>
<td>EPO-receptor</td>
<td>0.4 ± 0.06*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>6.2 ± 1.90*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>8.4 ± 1.03*</td>
</tr>
<tr>
<td>p-selectin</td>
<td>38.9 ± 7.9*</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>3.5 ± 0.19*</td>
</tr>
<tr>
<td>BAX</td>
<td>1.4 ± 0.09*</td>
</tr>
<tr>
<td>BCL-2</td>
<td>0.7 ± 0.03*</td>
</tr>
<tr>
<td>BAX/BCL-2 ratio</td>
<td>2.1 ± 0.22*</td>
</tr>
<tr>
<td>PAI-1</td>
<td>2.8 ± 0.53*</td>
</tr>
<tr>
<td>HO-1</td>
<td>15.8 ± 3.9*</td>
</tr>
</tbody>
</table>

Gene expression in the sham operated control group was set at 1. TNF: tumor necrosis factor; IL: interleukin; VCAM: vascular cell adhesion molecule; BAX: BCL-2 associated x protein; BCL: B-cell lymphoma; PAI: Plasminogen activator inhibitor; HO: heme oxygenase.

* significant at p<0.05 as compared to sham operated controls
† significant at p<0.05 as compared to BD
**PMN infiltration in the kidney**

To study influx of polymorphonuclear cells (PMNs) in the kidney, renal cryosections were stained with HIS-48 mAb. The number of positive cells per microscopic field was 3.3 ± 0.3 in sham operated controls, but was elevated to 7.4 ± 0.7 positive cells in the brain death group (P=0.001). Treatment with both EPO and cEPO diminished this effect to 5.1 ± 0.8 and 4.0 ± 1.2 positive cells, respectively, the effect reaching statistical significance at cEPO treatment EPO vs brain death control: p=0.06, cEPO: P=0.03) (Fig 3A-D). On histology, no structural changes were seen in the different groups.

**Figure 3** PMN infiltration in the kidney. 40x magnification.
Kidney function during IPK

Renal function was evaluated after the animal experiments by assessment of the kidney in an isolated perfused kidney model. Kidneys were evaluated during 90 minutes. Every 15 min., perfusate samples and urine production were collected to determine creatinine clearance. Area under the curve (AUC) was calculated for each experimental group. In kidneys from brain dead animals, creatinine clearance was clearly decreased compared with sham operated animals (P=0.035) (Fig 4). Treatment with both EPO and CEPO increased creatinine clearance to the level of sham operated animals (EPO: P=0.009, CEPO: P=0.016). Sodium concentration was measured in urine and perfusate, and fractional sodium excretion was calculated. As can be expected in an isolated perfused kidney model (27), fractional sodium excretion was increased to 20-30% of that in vivo, but no significant differences were found between the experimental groups.

Figure 4  Kidney function during reperfusion in the Isolated Perfused Kidney model
In the present study, we demonstrated the beneficial effects on the kidney of EPO and cEPO treatment in brain death. The effects can be divided into two categories.

In the first place, we examined the anti-inflammatory and endothelium preserving capacities of cEPO and EPO. EPO treatment reduced the expression of VCAM-1, an adhesion molecule that regulates leucocyte migration from the blood into tissues. Also, subsequent leucocyte migration was diminished, as shown by our staining for PMN infiltration in the kidney. cEPO, in addition, reduced the expression of p-selectin and e-selectin, both early adhesion molecules mediating the initial attachment of leucocytes to endothelial cells (34). Also, cEPO directly attenuated the expression of IL-1β and IL-6, both major pro-inflammatory cytokines which stimulate immune cells, promote leucocyte adhesion to the endothelium and thrombosis (35;36). Compared to EPO, cEPO treatment caused a lower PMN infiltration in the kidney, which can be explained by the lower grade of inflammation found with the reduced expression levels of cytokines and selectines.

Secondly, the effect of cEPO and EPO on kidney function after brain death was evaluated using an isolated perfused kidney model. In this model, creatinine clearance can be determined very accurately, without interference of recipient related variables when a model of kidney transplantation is used. Our study reveals that brain death causes a significant decrease in kidney function. This phenomenon has not been described before, as the determination of kidney function in the DBD donor is complicated due to factors as hemodynamic instability and its treatment with fluids and inotropic medication, and the presence of brain injury related diabetes insipidus. Recently, Blasco et al however reported the incidence of a more than 20% increase of serum creatinine level between intensive care unit admission and graft harvest to be 41% in DBD donors (37). These results confirm our measurements at the end of the brain death experiments, when plasma creatinine is increased in all brain dead groups. Part of this 'in vivo' effect may be explained by a state of low hydration in all brain dead animals, as intravenous infusion requirement was based on mean arterial pressure and not on diuresis. In the IPK setup we were able to demonstrate the independent effect of brain death on kidney function. The treatment effect of either cEPO or EPO during brain death experiments could only be inferred indirectly: while urine creatinine concentrations are decreased in the treated groups suggesting an increased diuresis during brain death after treatment with cEPO and EPO, intravenous infusion requirement (defined as loss of haemodynamic stability) was not increased. However, the IPK setup, measuring true glomerular filtration rate, did show a complete neutralization of the brain death induced decrease in kidney function after treatment with both EPO and cEPO.

The decreased expression of pro-inflammatory cytokines and adhesion molecules as measured in this study indicates a protective effect of EPO and cEPO on the endothelium. In
contrast, we found no effect of EPO or cEPO treatment on tubular cells: Alanine aminopeptidase (AAP) levels, a urinary marker for tubular damage, were not affected by EPO or cEPO administration, and the expression of kidney injury molecule-1 (KIM-1) which was highly increased in brain death, was not altered by EPO or cEPO either. Therefore, the improvement in kidney function as observed after EPO and cEPO administration is most likely caused by an improvement in endothelial and thus glomerular function of the kidney. The effects of EPO and cEPO administration in our model were observed after a single treatment and within a time period of several hours. This enlarges the clinical applicability and reduces the unwanted side effects of EPO. Parsa et al have shown that these effects take only place after a minimum of four days’ treatment with EPO (15). Treatment with cEPO was even more favourable, given its higher efficacy and the absence of erythropoietic and thrombotic activity (38).

In this model we pretreated BD rats. Following experiments are needed to investigate whether EPO and/or cEPO are capable of improving renal quality after BD induction by repairing existing damage. Some studies indicate the EPO and cEPO are not only protective but also stimulate repair mechanisms. Fiordaliso et al showed that cEPO administration given 5 min before reperfusion after a 40 min period of coronary artery ligation resulted in a significant reduction of cardiomyocyte loss and improved left ventricle function (23). In a model of cerebral injury, EPO administration up to six hours after occlusion of the middle cerebral artery attenuated the volume of cerebral infarction as much as 75% in rats (21).

- Conclusions -

In this study, we show that pretreatment with a single dose of cEPO, a modified derivative of EPO without erythropoietic properties, can reduce several inflammatory responses induced by brain death and can preserve vascular integrity. Treatment with cEPO proved to be more effective than EPO in the reduction of inflammatory parameters as measured in the brain dead donor. In addition, cEPO and EPO are able to preserve kidney function after brain death. We conclude that cEPO could be an interesting renoprotective candidate for clinical intervention during donor management and prior to retrieval followed by ischemia/reperfusion injury.
References


