Optical methods for the assessment of microvascular perfusion and oxygenation
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COMBINED LASER SPECKLE IMAGING AND PHOSPHORIMETRY FOR STUDYING THE ROLE OF RENAL HYPOPERFUSION IN THE DEVELOPMENT OF RENAL MICROCIRCULATORY DYSFUNCTION IN ENDOTOXEMIC RATS

Adapted from

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Intensive Care Medicine (in press)
*Equal contribution
The purpose of this study was to investigate the role of renal hypoperfusion in the development of microcirculatory dysfunction in endotoxemic rats. To this end, rats were randomized into 4 groups: a sham group (n=6); a lipopolysaccharide (LPS) group (n=6); a group in which LPS administration was followed by immediate fluid resuscitation which prevented the drop of renal blood flow (EARLY group) (n=6); and a group in which LPS administration was followed by delayed (i.e., a two-hour delay) fluid resuscitation (LATE group) (n=6). Renal blood flow was measured using a transit-time ultrasound flow probe. Microvascular perfusion and oxygenation distributions in the renal cortex were assessed using laser speckle imaging and phosphorimetry, respectively. IL-6, IL-10 and TNF-α were measured as markers of systemic inflammation. Furthermore, renal tissue samples were stained for leukocyte infiltration and iNOS expression in the kidney. We found that LPS infusion worsened both microvascular perfusion and oxygenation distributions. Fluid resuscitation improved the perfusion histograms but not the oxygenation histograms. Improvement of microvascular perfusion was more pronounced in the EARLY group compared to the LATE group. Serum cytokine levels decreased in the resuscitated groups with no difference between the EARLY and LATE groups. However, iNOS expression and leukocyte infiltration in the glomeruli were lower in the EARLY group compared to the LATE group. In conclusion, the prevention of endotoxemia-induced systemic hypotension by immediate fluid resuscitation (EARLY group) did not prevent systemic inflammatory activation (IL-6, IL-10, TNF-α) but did reduce renal inflammation (iNOS expression and glomerular leukocyte infiltration). However, it could not prevent reduced renal microvascular oxygenation.
INTRODUCTION

Acute kidney injury (AKI) is a common complication in septic patients [Uchino et al., 2005; Bagshaw et al., 2007]. The primary resuscitation strategy for these patients is fluid resuscitation, which is aimed at improving organ perfusion and oxygenation and thereby prevent (multiple) organ failure. For the kidney, however, the actual contribution of hypoperfusion to the pathogenesis of AKI has recently been challenged [Langenberg et al., 2005, 2007; Saotome et al., 2010]. In a model of hyperdynamic sepsis in sheep [Langenberg et al., 2007], AKI was associated with renal vasodilation and renal hyperemia and recovery of renal function was associated with relative vasoconstriction and decrease in renal blood flow. These findings contradict the widely accepted concept that renal hypoperfusion is the underlying cause for the development of sepsis-induced AKI. Contrastiingly, in animal models with lipopolysaccharide (LPS) as a key mediator of gram-negative bacterial endotoxemia, it has been reported that LPS-induced endotoxemia is associated with renal hypoperfusion and microvascular hypoxia [Johannes et al., 2006, 2009a]. In addition, the hypotensive conditions associated with endotoxemia were also suggested to participate in the activation of the systemic inflammatory response and consequent depression of renal perfusion and oxygenation [Nelson et al., 1988; Humer et al., 1996]. In this line, it is possible that even a brief period of renal hypoperfusion prior to the start of fluid resuscitation could result in an ischemia-reperfusion insult that injures the renal microcirculation and potentially leads to microvascular perfusion and oxygenation heterogeneities and ultimately to AKI [Le Dorze et al., 2009; Jang et al., 2009]. However, insight into the renal microvascular perfusion and oxygenation heterogeneity during endotoxemia and resuscitation is still lacking and the contribution of the systemic hypotension and renal hypoperfusion during endotoxemia to renal microcirculatory dysfunction and renal failure remains to be elucidated.

Endotoxemia has also been associated with an impaired ability to increase oxygen extraction, even in the presence of normal or elevated cardiac output and systemic oxygen delivery [Schumacker and Samsel, 1989; Bredle et al., 1989; Walley, 1996; Goldman et al., 2004]. This finding has led to the concept that oxygen consumption/supply dependency in sepsis is a direct result of regional rather than global oxygen delivery insufficiencies due to heterogeneity in microvascular perfusion and oxygenation. This may explain why organ failure continues to progress even after blood pressure and cardiac output are restored [Gattinoni et al., 1995; Dubin et al., 2009b].

Hence, while it has been shown that fluid resuscitation decreases mortality in septic shock [Rivers et al., 2001], controversy exists regarding the role of renal hypoperfusion (and thus the therapeutic value of fluid resuscitation) in sepsis-induced renal microcirculatory dysfunction. The purpose of the present study
was therefore to investigate the role of renal hypoperfusion in the development of microcirculatory dysfunction in endotoxemic rats. We hypothesized that prevention of endotoxemia-induced hypotension by immediate fluid resuscitation would prevent the development of renal microcirculatory dysfunction. To this end, we studied the efficacy of immediate fluid resuscitation to prevent the endotoxemia-induced systemic hypotension and renal hypoperfusion (compared to that of delayed fluid resuscitation) with respect to the protection of the renal microcirculation in a rat model of LPS-induced endotoxemia. Microvascular perfusion histograms in the renal cortex were assessed using laser speckle imaging (LSI), and microvascular oxygen tension histograms were assessed using phosphorimetry. Furthermore, IL-6, IL-10 and TNF-α were measured as markers of systemic inflammation and renal tissue samples were stained for leukocyte infiltration and iNOS expression in the kidney.

### METHODS

#### Animals

All experiments in this study were reviewed and approved by the Animal Research Committee of the Academic Medical Center at the University of Amsterdam, The Netherlands. Care and handling of the animals was performed in accordance with the guidelines of the Institutional Animal Care and Use Committees. Experiments were performed on 24 male Wistar rats (Harlan, Horst, the Netherlands) with a mean±SEM body weight of 351±27 g.

#### Surgical preparation

The rats were anesthetized with an intraperitoneal injection of 90 mg/kg ketamine (Nimatek®, Eurovet, Bladel, The Netherlands), 0.25 mg/kg medetomidine (Domitor®, Pfizer, New York, NY, USA) and 0.05 mg/kg atropine sulfate (Centrafarm, Etten-Leur, The Netherlands). After tracheotomy, the animals were mechanically ventilated with an FiO₂ of 0.4. Body temperature was maintained at 37±0.5 °C by external warming. The ventilator settings were adjusted to maintain an arterial pCO₂ between 35 and 40 mmHg. A polyethylene catheter was placed in the right carotid artery to monitor arterial blood pressure and heart rate. The right jugular vein was cannulated for continuous infusion of Ringer’s lactate (Baxter, Utrecht, The Netherlands) at a rate of 15 ml/kg/hour.

The left kidney was decapsulated and immobilized via a ~4-cm incision in the left flank. One perivascular transit-time flow probe (type 0.7 RB; Transonic Systems Inc., Ithaca, NY) was placed around the left renal artery and another flow probe was placed around the aorta just above the renal artery; both probes were connected to a flow meter (T206; Transonic Systems Inc., Ithaca, NY) for continuous measurement of renal blood flow (RBF) and aortic blood flow (AoBF),
respectively. Renal vascular resistance (RVR; dynes sec cm⁻³) was calculated as MAP/RBF×80. The left ureter was cannulated with a polyethylene catheter for urine collection.

After the surgical protocol was completed, one phosphorimetric fiber was placed 1 mm above the surface of the kidney for microvascular PO₂ measurements. The LSI device was placed approximately 40 cm above the surface of the kidney for the assessment of cortical microvascular perfusion as described below. Oxyphor G2 (Oxygen Enterprises, Ltd) was subsequently infused (6 mg/kg) intravenously for 5 minutes, followed by 30 minutes of stabilization.

**Experimental protocol**

The rats were randomized into four groups: 1) a sham operation group (SHAM, n=5); 2) an LPS control group (LPS, n=5); 3) an LPS group with immediate fluid resuscitation (EARLY, n=7); and 4) an LPS group with delayed fluid resuscitation (LATE, n=7).

Endotoxemia was induced by a 30-minute infusion of LPS (2.5 mg/kg, serotype 0127:B8, Sigma, The Netherlands). Immediate fluid resuscitation was started simultaneously with the LPS infusion by infusing a colloid solution (Voluven®, 6% HES 130/0.4; Fresenius Kabi Nederland, Schelle, Belgium) at a rate of 20 ml/kg/h in the first hour followed by 5 ml/kg/h for the remainder of the experimental protocol. This regimen was based on pilot experiments that demonstrated that this regime could prevent a drop in the mean arterial pressure and renal blood flow during LPS infusion. Delayed fluid resuscitation, in contrast, was started 120 min after the LPS infusion; the fluids and infusion rates were identical to that used in the immediate resuscitation regimen.

The experiments were terminated by infusing 1 ml of 3 M potassium chloride. Subsequently, the kidney was removed, weighed, placed in formalin, and fixed in paraffin blocks for immunohistochemical analysis.

**Blood gas measurements**

Arterial blood samples (0.5 ml) were drawn at three time points (ABL505 blood gas analyzer; Radiometer, Copenhagen, Denmark). The time points were 1) before infusion of LPS (baseline); 2) 120 min after infusion of LPS; and 3) 300 min after infusion of LPS. The withdrawn blood samples were replaced by the same volume of HES130/0.4 (Voluven®).

**Microvascular perfusion in the renal cortex**

LSI is a laser-based, full-field perfusion monitoring technique that provides pseudo-color images of a macroscopic field of view. The technique is based on the formation of an interference pattern (or speckle pattern) on a CCD camera when a diffuse surface (e.g., biological tissue) is imaged using highly coherent light (i.e.,
a laser or laser diode) for illumination. Movement of scattering particles (e.g., red blood cells within the penetration depth of the illumination light) will influence the speckle pattern over time. Hence, tissue perfusion in the field of view results in fluctuations in the speckle pattern; by integrating these fluctuations over the CCD camera exposure time, pixel areas with a high level of perfusion will have lower contrast, and pixel areas with a low level of perfusion will have higher contrast. The speckle contrast, defined as the ratio of the standard deviation to the mean grayscale intensity in a small window (5x5 pixels), is related to the mean velocity of the red blood cells in this window. Therefore, LSI is capable of producing real-time images of tissue microvascular perfusion, as we validated in a recent study [Bezemer et al., 2010b].

For LSI measurements, a commercially available system was used (Moor Instruments, Devon, UK). A 785-nm class 1 laser diode was employed for illumination of the tissue to a depth of approximately 1 mm. Directly reflected light by the tissue surface was blocked by a tunable polarization filter placed in front of the lens system since it was not scattered by flowing red blood cells and therefore contained no information on tissue perfusion. Laser speckle images were acquired using a 576×768 pixels grayscale CCD camera at a frame rate of 25 Hz (exposure time of 4 ms) and converted to pseudo-color images where the contrast (thus the level of perfusion) was scaled from blue (low perfusion) to red (high perfusion) (Figure 1). The distribution of cortical flow velocities are depicted in LSI perfusion histograms [Bezemer et al., 2010b]. The lens system allowed a variable zoom, ranging from 0.6×0.8 cm (corresponding to 10 µm/pixel) to 9×12 cm at a working distance of 15-45 cm, and focus optimization. For LSI of the rat kidney, the field of view was set to ~1.8×2.4 cm (corresponding to ~30 µm/pixel). Using a 5×5 pixel window to calculate speckle contrast, the maximal image resolution was ~150 µm/pixel area.

**Microvascular oxygenation in the renal cortex**

Renal cortical microvascular PO$_2$ (µPO$_2$) was measured by phosphorimetry. Phosphorimetry is based on oxygen-dependent quenching of the phosphorescence of the phosphorescent dye Oxyphor G2 (a two-layer glutamate dendrimer of tetra-(4-carboxy-phenyl) benzoporphyrin) (Oxygen Enterprises, Philadelphia, PA). The lifetime of the phosphorescence decay trace is inversely related to the µPO$_2$ by the Stern-Volmer relation; thus, recovering the phosphorescence lifetime distribution allows for the quantitative measurement of µPO$_2$ histograms. The limited optical penetration depth at the excitation wavelength of Oxyphor G2 (440 nm) allows for phosphorimetric measurements in the microcirculation of the renal cortex. Details of the technique have previously been published elsewhere [Lo et al., 1997; Johannes et al., 2006; Bezemer et al., 2010c].

**Renal function**

As an indicator of renal function, creatinine clearance (Clearcrea [(ml/min)] was assessed as an index of the glomerular filtration rate. Calculations of the clearance were done using the standard formula: Clear crea=(Ucrea×V)/Pcrea. The concentrations of creatinine in urine and plasma were determined by colorimetric methods.

**Systemic inflammation**

![Figure 1. Typical laser speckle imaging perfusion maps; perfusion is displayed on a 16-level color palette scaled from blue (low perfusion) to red (high perfusion) in a rat receiving an LPS infusion (t=30 min) followed by delayed fluid resuscitation (t=300 min).](image_url)
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**Systemic inflammation**

Plasma concentrations of interleukins (IL)-6 and -10 and tumor necrosis factor (TNF)-$\alpha$ were determined using rat singleplex bead kits (Invitrogen, Breda, The Netherlands) and read with a BioRad Bioplex 100 (BioRad, Hercules, CA).

**Renal inflammation**

Induction of inducible nitric oxide synthase (iNOS) and infiltration of leukocytes into the kidney are considered major events in the development of septic AKI. Therefore, iNOS immunostaining and anti-myeloperoxidase (MPO) staining were performed.

For this study, the kidney tissues were fixed in 4% formalin, embedded in paraffin, and prepared as described previously [Legrand et al., 2009]. Both the intensity and the distribution of specific iNOS staining were scored using the HSCORE ($\text{HSCORE} = S \times P_i$ (i+1), where $i$ is the intensity score and $P_i$ is the corresponding percentage of the cells). We evaluated the MPO reaction in the glomerular and peritubular areas from renal samples (randomized and blinded to the analyst) from which 315 glomerular and peritubular areas were selected under a light microscope at a magnification of 400x. If no leukocytes could be seen in the glomeruli, the sample was assigned a score of 0; if leukocytes could be seen in the glomeruli, the sample was assigned a score of 1.

**Statistical analysis**

All data are presented as the mean±SEM. The phosphorescence decay curves were analyzed using software programmed in Labview 6.1 (National Instruments, Austin, TX, USA). Statistical analysis was performed using GraphPad Prism, version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). The assumption of
normality was checked using the Kolmogorov-Smirnov normality test. Analysis of variance (ANOVA) with Bonferroni post-hoc testing was used for inter-group comparisons (Figures 5, 6, 7, and 8) and repeated measures ANOVA with Bonferroni post-hoc testing was used for intra-group comparisons (Figure 2). The recovered microcirculatory perfusion and oxygenation histograms were compared using Kolmogorov-Smirnov’s test (Figure 3 and 4). This test converts each histogram into a cumulative histogram and then finds the maximum difference (D) between a set of cumulative histograms. For a given number of histogram bins, the D-values correspond to p-values, where p<0.05 indicates that the two histograms are significantly shifted with respect to one another. For the 16-bin perfusion histograms, p<0.05 if D>0.2947; for the 21-bin pO₂ histograms, p<0.05 if D>0.2586. For all analyses, p<0.05 was considered statistically significant.

RESULTS

All groups received 15 ml/kg/hour Ringer’s lactate solution during the entire protocol. In addition, the EARLY group and the LATE group received 20 ml/kg/hour of HES solution in the first hour of resuscitation, followed by 5 ml/kg/hour the remaining duration of the protocol. Hence, in total, the rats in the EARLY group received 40 ml/kg in 300 min and the rats in the LATE group received 30 ml/kg in 300 min.

Systemic hemodynamic parameters

Arterial blood gas values are reported in Table 1. The systemic hemodynamic parameters are shown in Figure 2. Infusion of LPS induced a biphasic reduction in MAP in which MAP initially decreased to ~65% of the baseline (first 30 min), then increased to approximately baseline level (next 3 hours), and ultimately decreased to ~55% of baseline at the end of the protocol (LPS group and LATE group). In the EARLY group, fluid resuscitation prevented the initial decrease in MAP in the first phase, but in the second phase, MAP decreased to ~80% of baseline. Delayed fluid resuscitation in the LATE group improved MAP from ~55% (LPS group) to ~70% of baseline at the end of the protocol.

Infusion of LPS induced an immediate reduction in AoBF to ~40% of baseline in the LPS group and the LATE group. In the EARLY group, fluid resuscitation resulted in a ~30% elevated AoBF. Delayed fluid resuscitation in the LATE group first increased AoBF to above baseline after which it decreased to approximately baseline level.

Renal blood flow

RVR increased rapidly in the LPS and the LATE groups, while it remained stable in the SHAM and the EARLY groups. Late resuscitation was able to restore RVR back to its baseline level. RBF followed an inverted pattern and decreased sharply after the infusion of LPS to reach ~20% of baseline at the end of the protocol. The
fall in RBF was prevented in the EARLY group, in which RBF first increased above its baseline level and then slightly decreased to ~85% of baseline at the end of the protocol. In the LATE group, fluid resuscitation temporarily restored RBF to its baseline level, after which it decreased to ~70% of baseline.

**Renal microcirculatory parameters**

Renal microvascular perfusion and oxygenation histograms in the renal cortex as measured using LSI and phosphorimetry before and after 30-min infusion of LPS and after fluid resuscitation are depicted in Figures 3 and 4, respectively.

**Renal microvascular perfusion**

LPS infusion induced a left shift in the perfusion histogram in the LPS group (D=0.7588, p<0.05) and the LATE group (D=0.7682, p<0.05). Immediate fluid resuscitation prevented this left shift (D=0.0929, p>0.05). At the end of the protocol, the perfusion histograms in the EARLY group (D=0.6920, p<0.05) and the LATE group (D=0.4720, p<0.05) were right-shifted with respect to the histogram in the LPS group. Additionally, the perfusion histograms were significantly more right-shifted in the EARLY group than in the LATE group (D=0.4444, p<0.05).

### Table 1. Arterial blood gas values at t=0 min (baseline) and t=300 min (end of protocol). *p<0.05 vs SHAM, †p<0.05 vs LPS, ‡p<0.05 vs EARLY.*

<table>
<thead>
<tr>
<th></th>
<th>t=0 min</th>
<th>t=300 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PaO₂ (mmHg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>235±16</td>
<td>235±11</td>
</tr>
<tr>
<td>LPS</td>
<td>219±7</td>
<td>222±10</td>
</tr>
<tr>
<td>EARLY</td>
<td>248±8</td>
<td>200±22</td>
</tr>
<tr>
<td>LATE</td>
<td>225±21</td>
<td>233±12</td>
</tr>
<tr>
<td><strong>PaCO₂ (mmHg)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>34±3</td>
<td>34±2</td>
</tr>
<tr>
<td>LPS</td>
<td>37±3</td>
<td>30±6</td>
</tr>
<tr>
<td>EARLY</td>
<td>35±3</td>
<td>37±5</td>
</tr>
<tr>
<td>LATE</td>
<td>33±2</td>
<td>31±2</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>7.36±0.03</td>
<td>7.29±0.02</td>
</tr>
<tr>
<td>LPS</td>
<td>7.31±0.01</td>
<td>7.02±0.03 †</td>
</tr>
<tr>
<td>EARLY</td>
<td>7.36±0.02</td>
<td>7.20±0.05 ‡</td>
</tr>
<tr>
<td>LATE</td>
<td>7.37±0.02</td>
<td>7.15±0.02 ‡</td>
</tr>
<tr>
<td><strong>HCO₃⁻ (mmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>18.0±1.1</td>
<td>16.2±0.2</td>
</tr>
<tr>
<td>LPS</td>
<td>20.2±0.56</td>
<td>12.4±4.4</td>
</tr>
<tr>
<td>EARLY</td>
<td>19.1±0.7</td>
<td>13.8±1.7</td>
</tr>
<tr>
<td>LATE</td>
<td>18.8±0.5</td>
<td>10.4±0.6 ‡</td>
</tr>
<tr>
<td><strong>Base deficit (mmol/L)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHAM</td>
<td>6.0±1.1</td>
<td>8.8±0.7</td>
</tr>
<tr>
<td>LPS</td>
<td>4.8±0.6</td>
<td>18.6±3.6 ‡</td>
</tr>
<tr>
<td>EARLY</td>
<td>4.7±0.6</td>
<td>13.3±1.4 ‡</td>
</tr>
<tr>
<td>LATE</td>
<td>4.7±0.5</td>
<td>17.3±0.9 SE</td>
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</table>
Figure 2. Mean arterial pressure (MAP, panel A), aortic blood flow (AoBF, panel B), renal blood flow (RBF, panel C), and renal vascular resistance (RVR, Panel D) during LPS infusion (t=0-30 min) and fluid resuscitation. The LPS group received no fluid resuscitation. In the EARLY group, fluid resuscitation was started simultaneously with LPS infusion. In the LATE group, fluid resuscitation was started with a 2-hour delay. *p<0.05, LPS vs. LATE; #p<0.05, LATE vs. EARLY; ¤p<0.05, LPS vs. EARLY.
Renal microvascular oxygenation

In contrast to its effect on microvascular perfusion histograms, LPS infusion did not immediately affect the microvascular PO$_2$ histograms in the LPS group ($D=0.1918$, $p>0.05$), the EARLY group ($D=0.1908$, $p>0.05$), or the LATE group ($D=0.1798$, $p>0.05$). At the end of the protocol, however, the PO$_2$ histograms were all left-shifted with respect to baseline: $D=0.4461$ and $p<0.05$ in the LPS group, $D=0.3898$ and $p<0.05$ in the EARLY group, and $D=0.2764$ and $p<0.05$ in the LATE group. No significant differences in oxygenation histograms were found at the end of the protocol between the LPS group, the EARLY group, or the LATE group ($D<0.1438$, $p>0.05$).
While all rats in the LPS group suffered from AKI as reflected by anuria, fluid resuscitation only partially prevented AKI. Creatinine clearance rates in the EARLY and LATE groups did not differ but were statistically lower compared to the rates in the SHAM group (respectively 0.96±0.10 and 0.72±0.09 ml/min versus 1.93±0.47 ml/min, p<0.01).

Systemic inflammation

Infusion of LPS induced a marked increase in IL-6, IL-10 and TNF-α plasma levels (Figure 5). Fluid resuscitation partially prevented the increase in IL-6, IL-10 and TNF-α plasma levels. The efficacy of fluid resuscitation in reducing plasma levels
of these pro-inflammatory cytokines, however, was not dependent on its timing (i.e., no statistically significant differences the EARLY group and the LATE group).

**Renal inflammation**

LPS infusion induced leukocyte infiltration in both peritubular and glomerular areas (Figure 6) and iNOS expression (Figure 7) as shown by MPO and iNOS staining, respectively. While delayed fluid resuscitation was not effective in

![Figure 5. Plasma IL-6, IL-10 and TNF-α level in the four experimental groups at the end of the protocol. The LPS group received no fluid resuscitation. In the EARLY group, fluid resuscitation was started simultaneously with LPS infusion. In the LATE group, fluid resuscitation was started with a 2-hour delay. LPS induced an increase in IL-6, IL-10, and TNF-α while immediate and delayed fluid resuscitation reduced their concentration to a similar extent.](image)

![Figure 6. MPO-stained leukocytes in the peritubular areas and glomeruli in the four experimental groups. Immediate and delayed fluid resuscitation could partially prevent leukocyte infiltration in the peritubular areas and, additionally, immediate fluid resuscitation could partially prevent leukocyte infiltration in the glomeruli after LPS infusion.](image)

![Figure 7. iNOS expression in the four experimental groups. Immediate fluid resuscitation partially prevented excessive iNOS expression after LPS infusion.](image)
reducing leukocyte infiltration and iNOS expression, these parameters were significantly improved by preventing the endotoxemia-induced hypotension and renal hypoperfusion with immediate fluid resuscitation.

**DISCUSSION AND CONCLUSIONS**

The purpose of the present study was to investigate the role of renal hypoperfusion in the development of microcirculatory dysfunction in endotoxemic rats and to test the hypothesis that prevention of endotoxemia-induced hypotension by immediate fluid resuscitation would prevent the development of renal microcirculatory dysfunction. To this end, we developed an animal model in which the initial endotoxemia-induced reduction in renal blood flow was prevented by applying an immediate fluid resuscitation regimen in an LPS-induced rat model of endotoxemic shock. We have shown that prevention of renal macrovascular hypoperfusion could not fully protect renal microcirculatory dysfunction. This indicates that endotoxemia-induced renal microcirculatory dysfunction can exist under conditions of maintained macrovascular perfusion. We have furthermore shown that prevention of hypotension by immediate fluid resuscitation could reduce renal inflammatory activation, but not systemic inflammatory activation, suggesting that endotoxemia-induced hypotension prior to fluid resuscitation leads to an ischemia-reperfusion insult that potentially leads to the activation of renal inflammation.

The role of renal hypoperfusion in the development of AKI in septic patients remains a matter of debate. The question whether to give these patients large fluid volume is of paramount importance given the associated increase risk of mortality in patients with AKI and a positive fluid balance [Payen et al., 2008; Bouchard et al., 2009]. Langenberg et al. have previously reported that AKI could develop despite an increase in the renal blood flow of resuscitated septic sheep [Langenberg et al., 2007]. The same group has recently shown that temporary renal hypoperfusion with a reduction in renal blood flow to 80% of baseline for two hours did not result in alterations in renal function or the histological features of renal injury [Saotome et al., 2010]. Johannes et al. found that severe mechanical reduction of renal blood flow did not lead to renal microcirculatory hypoxia. However, they also discovered that an LPS-induced reduction in renal blood flow led to hypoxic microcirculatory areas in the renal cortex [Johannes et al., 2009a]. Along these lines, the microcirculation of the renal cortex has been shown to be the severely injured in animal models of sepsis [Di Giantomasso et al., 2003; Johannes et al., 2009a,b,c; Dyson et al., 2011]. However, none of these studies was designed to isolate those effects of LPS directly related to hemodynamic alterations from those related to a direct inflammatory response. In the present study, in contrast, we could show that endotoxemia-induced renal microcirculatory failure can exist while macrovascular perfusion is maintained.
Other animal studies have shown that severe sepsis is characterized by a reduction in functional capillary density and an increase in blood flow heterogeneity. Goldman et al. showed that increased heterogeneity in skeletal muscle microcirculation could lead to a mismatch between local oxygen supply and demand, which resulted in an increased dependency of tissue oxygen utilization on the microcirculatory oxygen supply [Goldman et al., 2004]. Therefore, increasing systemic oxygen delivery to normal or supernormal levels may not improve tissue oxygenation if the microcirculatory perfusion and oxygen supply are not properly distributed in the tissue [Cabrales et al., 2005; Dubin et al., 2009b; Le Dorze et al., 2009; Jhanji et al., 2009]. Whether this phenomenon of microcirculatory perfusion heterogeneity in sepsis could apply to the kidney has not been considered previously.

We assessed microvascular perfusion distributions in the renal cortex using LSI and measured cortical microcirculatory oxygenation distributions using phosphorimetry. The advantage of LSI is its ability to provide on-line mapping of the cortical microcirculatory perfusion with high spatial and temporal resolution and to measure perfusion distribution/re-distribution in time [Bezemer et al., 2010b]. Using these techniques, we found that immediate fluid resuscitation, which was designed to prevent the endotoxemia-induced drop in renal blood flow, was not able to completely prevent the occurrence of renal microcirculatory hypoperfusion and hypoxia. However, by means of histogram analysis we identified that fluid resuscitation did reduce the extent of hypoxia as reflected by the reduced fraction of the first histogram bins.

There is no doubt that fluid resuscitation should be applied as early as possible to prevent AKI. However, our results suggest that completely avoiding renal macrohemodynamic failure does not prevent the development of microcirculatory failure. Hence, the macrohemodynamic increase in renal blood flow should be differentiated from the recruitment of downstream microcirculation. Sepsis-induced microcirculatory dysfunction has been linked to several factors, including vasoconstrictor and vasodilator mediator imbalances, red blood cell and platelet aggregation, and the activation of local inflammation [Kinsey et al., 2008; Le Dorze et al., 2009; Jang et al., 2009]. Hence, sepsis affects several aspects required for proper microvascular function, thereby resulting in significant disturbances in capillary perfusion. Indeed, the results of our study show that renal microcirculatory hypoperfusion and renal hypoxia occur during endotoxemia. In addition, this microcirculatory failure appears to be relatively independent from systemic and renal macrohemodynamics, but arises from intrarenal mechanisms that may be associated with heterogeneity in the microcirculation.

A mechanism potentially responsible for the disassociation between macro- and microcirculatory parameters could be the inadequate resolution of inflammatory activation by just improving systemic hemodynamics [Kinsey et al., 2008; Le Dorze et al., 2009; Jang et al., 2009]. Despite the prevention of hypoperfusion, immediate fluid resuscitation was unable to prevent systemic inflammation. This
is possibly combined with a suboptimal fluid composition leading to a disturbed plasma ion levels (e.g., hyperchloremia) and reduced oxygen carrying capacity. Hence, the hypoperfusion and subsequent reperfusion in the early phase of endotoxemia probably contributes to the pathogenesis of endotoxemia-related AKI by forming an additional hit besides the endotoxemia-induced inflammation.

In the present study, systemic and renal inflammation responded differently to the applied fluid administration protocols. Inflammation in the renal cortex appeared to be partially prevented by immediate fluid resuscitation, as evidenced by both a decrease in leukocyte infiltration and a decrease in iNOS expression in the EARLY group compared to the LATE group. However, neither immediate nor delayed fluid resuscitation was able to prevent activation of the systemic inflammatory response, as indicated by similar increases in plasma cytokine levels (IL-6, IL-10, and TNF-α). The present study, however, does not allow differentiation between those effects directly related to microcirculatory perfusion and oxygenation deficits and those related to the inflammatory response as immediate fluid resuscitation did not translate into an improvement in creatinine clearance in this short study protocol.

We are aware that our study suffers from several limitations inherent to the use of an animal model of endotoxemia. First, endotoxemic models may not reflect all the situations encountered in human sepsis and may lack relevance in gram-positive sepsis. However, it is a calibrated and reproducible model of acute inflammation that involves similar pathways and thus allows us to study the roles of renal hypoperfusion and microcirculatory dysfunction in the pathogenesis of endotoxemia-induced AKI. In this model flow histograms slightly narrowed and the oxygenation histograms slightly decreased in time as seen in the SHAM group. This possibly adds up to the changes associated by LPS and could contribute to the lack of full recovery of the flow and oxygenation histograms. However, we could still clearly observe the effects of LPS and the differences between immediate and delayed fluid resuscitation. Thus, although the model may be slightly instable over time, it still allows delineation between those effects of LPS directly related to hemodynamic alterations and those related to a direct inflammatory response as reflected by the differences between the EARLY and the LATE group. Extrapolation of this model to clinical scenarios in terms of treatment strategies should be made with utmost caution. Instead, our study should be regarded as adding to our understanding of the factors contributing to renal microcirculator failure in sepsis. Furthermore, fluid resuscitation here was very early in comparison to resuscitation in clinical scenarios. Another point of concern is that the immediate fluid resuscitation regimen might have led to an altered LPS disposition in the kidney compared to the delayed resuscitation regimen. However, the applied resuscitation protocols were required to appropriately address the question whether preventing the endotoxemia-induced renal hypoperfusion would prevent renal microcirculatory failure and. Although
fluid resuscitation restored urine output in both resuscitated groups, creatinine clearance remained below baseline level and whether creatinine clearance would improve in time in either of the resuscitation groups remains unidentified due to the short duration of the study protocol. Therefore, the present study only allows assessment of the hemodynamic- and inflammation-related effects of LPS in this short-term rat model of acute endotoxemia. Second, hydroxyl ethyl starches (HES)-based resuscitation strategies are controversial [Boussekey et al., 2010] and an effect of the fluid on kidney injury could not be excluded in the present study. However, only the old generation of high molecular weight HES molecules has been reported to be associated with acute renal failure in a dose-dependent fashion. There is no evidence for such an association with the 130/0.4 HES we used in this study. In contrast, low molecular weight HES molecules have been shown to have protective effects on microcirculation [Hoffman et al., 2002; Inan et al., 2009]. However, in the present study fluid resuscitation with a (HES)-based solution led to a reduction of the renal microcirculatory failure and inflammation compared to the non-resuscitated group. Third, changes in creatinine clearance as an index of glomerular filtration should only be regarded as a gross indicator of renal function in sepsis, which is an accepted indicator of renal function as described by the AKIN and RIFLE criteria. As more sensitive markers would be desirable, novel biomarkers are currently under investigation (e.g., neutrophil gelatinase-associated lipocalin and fatty acid binding protein), but the significance of their detection in the context of endotoxemia is a matter of concern [Martensson et al., 2010; Bagshaw et al., 2010]. Fourth, without additional data on mitochondrial function or cell viability it is unclear whether the observed alterations in the renal microcirculation were a primary or secondary cause of renal failure.

In conclusion, the present study has shown that LPS-induced endotoxemia can induce alterations in microvascular perfusion and oxygenation in the renal cortex in rats, which appear to be weakly dependent on systemic and renal macrohemodynamics. Prevention of endotoxemia-induced hypotension by immediate fluid resuscitation did not prevent systemic inflammation activation (as indicated by IL-6, IL-10, and TNF-α plasma levels) but did reduce renal inflammation (as indicated by iNOS expression and glomerular leukocyte infiltration). However, this did not result in improved renal microcirculatory function. This microcirculatory failure may partially explain why renal failure progresses even after (immediate) correction of endotoxemic hypotension.