High tidal volume mechanical ventilation during sepsis causes kidney apoptosis and decreased kidney function without significant lung injury.

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Abstract

Introduction: Septic acute kidney injury (AKI) is associated with increased mortality rates compared to non-septic AKI. Besides being an independent risk factor for increased mortality rates in septic AKI, mechanical ventilation (MV) also contributes to the development of AKI. The pathophysiology of septic AKI remains largely unknown, but recently renal apoptosis has been implicated. The present study was undertaken to investigate the role of MV in AKI during sepsis.

Methods: Sepsis was induced in 30, male Spraque-Dawley rats by cecal ligation and puncture. Six rats served as healthy controls. Animals were randomly assigned to three groups: 1) low tidal volume ($V_t$) of 6 ml/kg and positive end-expiratory pressure (PEEP) of 5 cm H$_2$O (LV$_t$), 2) high $V_t$ of 15 ml/kg and PEEP of 3 cm H$_2$O (HV$_t$), and 3) non-ventilated controls. Rats were ventilated for 4 hrs and macrophage inflammatory protein-2 (MIP-2), interleukin-6 and tumor necrosis factor-$\alpha$ were measured in plasma. The endothelial markers intercellular adhesion molecule-1 and plasminogen activator inhibitor-1 were measured in plasma and kidney homogenates. Renal function was measured and lung and kidney injury were assessed. Apoptosis was quantified using TUNEL staining.

Results: A significant increase in renal apoptosis was observed in the HV$_t$ group as compared to the LV$_t$ group and the non-ventilated sepsis group. This occurred in the absence of lung injury or MV-induced systemic inflammation. No signs of endothelial activation were present. Renal apoptosis was more profound in the renal medulla as compared to the cortex. The increased apoptosis was associated with decreased kidney function.

Conclusions: This study shows that non-injurious MV during sepsis induces kidney apoptosis and impairs kidney function, independent of lung injury. Although the exact mechanism by which MV during sepsis causes apoptosis remains unclear, this is likely a multi-factorial process involving alterations in local rather than global renal hemodynamics.
**Introduction**

Acute kidney injury (AKI) is a common complication of sepsis and is considered to be an independent risk factor for mortality.\(^1,2\) Various mechanisms contribute to the pathogenesis of septic AKI although the exact mechanisms remain unknown.\(^3-7\) Mechanical ventilation (MV) is an independent risk factor for mortality in septic AKI; MV can contribute to the development of AKI.\(^8,9\) A number of putative mechanisms have been proposed to explain this impact of MV including its effect on hemodynamics, inflammation, endothelial activation and apoptosis,\(^11\) but to date, very few studies have focused on these latter mechanisms.\(^11,12\)

A role for apoptosis in the pathogenesis of septic organ failure including AKI has been proposed.\(^13-15\) Within 3 hrs after the induction of sepsis, early phase pro-apoptotic proteins were detected in renal tubular cells of sheep.\(^3,15\) A recent clinical study showed increased apoptosis in renal biopsy specimens taken within 30 minutes following death in patients with sepsis induced AKI.\(^6\) However, experimental data on the role of MV in the induction of apoptosis and AKI during sepsis is lacking. O’Mahony et al. showed increased plasma creatinine levels and histological protein accumulation in the collecting tubules after MV following intraperitoneal lipopolysaccharide (LPS).\(^16\) This was associated with increased plasma cytokine levels. The authors concluded that MV enhances the systemic inflammatory response leading to extrapulmonary organ injury, including AKI, but did not investigate a role for apoptosis.\(^16\)

The present study was undertaken to investigate the role of MV in the pathophysiology of septic AKI. After induction of sepsis by cecal ligation and puncture we measured systemic inflammatory mediator levels, endothelial activation and kidney apoptosis in rats subjected to different ventilation strategies. We hypothesized that high tidal volume MV in septic rats induces systemic inflammation and endothelial cell activation associated with renal apoptosis and decreased kidney function.
Materials and methods

Animal Preparation.

Animals were treated according to the Canadian national guidelines and with approval of the Institutional Animal Care Committee of St Michael’s Hospital. Sprague Dawley rats (Charles Rivers, St Constan, QC, Canada) weighing 290 - 320g were anesthetized with xylazine (Bayer, Toronto, ON, Canada) 10 mg/kg and ketamine (Bimeda-MTC, Cambridge, ON, Canada) 100 mg/kg given intraperitoneally. Sepsis was induced using a modification of the cecal ligation and perforation technique described by Chaudry et al.\textsuperscript{17} and Bohnen et al.\textsuperscript{18} With the animals spontaneously breathing 40% oxygen, a laparotomy through a midline incision using an aseptic technique was performed. The cecum was ligated just below the ileocecal valve with 3-0 silk ligature, so that intestinal continuity was maintained. Using a 14-Gauge needle, the cecum was perforated in two locations, 1 cm apart, on the antimesenteric surface of the cecum, and the cecum was gently compressed until feces were extruded. The bowel was then returned to the abdomen and the incision was closed using 4-0 silk ligature for both the muscle layer and skin. Subsequently, rats received 3 ml/100g 0.9% saline in the scruff of the neck and buprenorphine 30 ug/kg subcutaneously (Schering-Plough, Hertfordshire, UK). The rats breathed 40% oxygen until recovery from anesthesia, and then were placed back in a cage with free access to food and water. Eight hours after surgery, rats received a 3 ml/100g 0.9% NaCl i.p.

Twenty-four hours after surgery rats randomized to MV were anesthetized with xylazine 10 mg/kg and ketamine 100 mg/kg given intraperitoneally. Rats were then placed supine on a heating pad to maintain body temperature at 37-37.5°C. A tracheostomy was performed and a cannula (14 gauge) was inserted into the trachea. Rats were connected to a ventilator and ventilated with a tidal volume ($V_t$) of 6 ml/kg and a positive end-expiratory pressure (PEEP) of 5 cm H$_2$O until randomization. Anesthesia was maintained with xylazine 1 mg/kg/h, ketamine 20 mg/kg/h intravenously; muscle relaxation was achieved by intravenous administration of pancuronium bromide (Sabex Inc, QC, Canada) 0.6 mg/kg/h. The bladder was catheterized using a transabdominal approach for collection of urine. For blood sampling and arterial blood pressure measurements, a catheter was inserted into the right carotid artery. All rats received a continuous infusion of normal saline (10 ml/kg/h) via the tail vein to keep mean arterial pressure >60 mmHg. All rats received the same amount of fluid during MV.
Experimental Protocol.
Septic animals were randomly assigned to three groups (n=10 per group): 1) low tidal volume (VT) of 6 ml/kg and PEEP of 5 cm H 2O (LV), 2) high VT of 15 ml/kg and PEEP 3 cm H 2O (HV), and 3) non-ventilated controls (sepsis). Six rats served as healthy controls (controls). Rats were ventilated for 4 hrs. The ventilatory settings were selected to maintain similar mean airway pressures in both ventilated groups. Normocapnia was maintained by adjusting respiratory rate. Inspiratory:expiratory ratio was set to 1:2, and the fraction of inspired oxygen was set at 0.4. Arterial blood samples were taken 30 minutes after randomization and every hour for blood gas analysis (Ciba Corning Model 248 blood gas analyser, Corning Medical, Medfield, MA, USA).

After 3 hrs of mechanical ventilation, bladders were emptied and urine samples were collected during the last hour of the experimental protocol. At the end of the experiment a blood sample was taken and animals were sacrificed with an overdose of anestheisa. Non ventilated controls were sacrificed 28 hrs after induction of sepsis. Kidneys were harvested for histological examination. Parts of the organs were snap frozen and stored at -80°C for further analysis. Part of the lung was dried to determine lung wet to dry weight (W/D) ratio. Creatinine clearance was calculated using the formula $U_C V / P_C$, where $U_C$ represents the urine creatinine concentration (mg/ml), $V$ is the urine flow (ml/min) and $P_C$ is the plasma creatinine concentration. Technically, it was not possible to collect urine in spontaneously breathing control animals without subjecting the rats to anesthesia and subsequent mechanical ventilation.

Measurements.
Cytokines. Plasma macrophage inflammatory protein-2 (MIP-2), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured in plasma by enzyme linked immunosorbent assay (ELISA) (Biosource, Camarillo, CA, USA) in healthy control animals, 24 hours after the induction of sepsis, and in septic animals after 4 hrs of MV. Lower detection limits for MIP-2, IL-6 and TNF-α are 1 pg/ml, 8 pg/ml and 4 pg/ml respectively.

ICAM-1, active PAI-1. Intercellular adhesion molecule-1 (ICAM-1) (R&D Systems Inc, Minneapolis, MN, USA) and active plasminogen activator inhibitor-1 (PAI-1) (Innovative Research Inc, Southfield, MI, USA) were measured in plasma and kidney homogenates using ELISA kits, specific for rat, according to manufacturer guidelines.
Endothelin-1. Endothelin-1 (ET-1) levels in plasma and kidney homogenates were measured using a human chemiluminescent ELISA (R&D Systems Inc.), shown to be cross reactive with rat endothelin-1. Total protein concentration in kidney homogenates was determined with a colorimetric ELISA assay, according to the protocol provided by the manufacturer (Bio-Rad Laboratories, Inc, Hercules, CA) using bovine serum albumin to construct a standard curve.

Histology.
A pathologist, blinded as to the experimental history of the specimens, performed a quantitative morphometric analysis of lung injury using a scoring system we have used previously\textsuperscript{19} and of kidney injury using a scoring system that included tubular dilatation, presence of intra-tubular debris, vacuolization of tubular epithelium cells and loss of brush border membrane integrity.\textsuperscript{20}

Apoptosis (TUNEL Assay).
Apoptotic cells were detected using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for in-situ end labeling, adapted to an automated in-situ hybridization instrument (Discovery \textsuperscript{TM} Ventana Medical Systems, Inc.Tuscon, AZ. USA) as done previously.\textsuperscript{21} As per Discovery protocol, the instrument used 5\textmu m thick deparaffinized tissue sections mounted on positive charged glass slides, with subsequent digestion with Protease I (Ventana Medical Systems, AZ. USA) digestion for 12 minutes. The assay uses recombinant terminal deoxynucleotidyl transferase (Tdt) (Invitrogen Corporation, CA, USA.) for adding homo-polymer tails to the 3’ ends of cleaved DNA, characteristic in cells undergoing programed cell death. Biotin 16-dUTP (Roche Diagnostics, Basel Switzerland) was the labeled nucleotide used for this reaction. Colorimetric visualization using avidin-horse radish peroxidase and 3,3’-diaminobenzidine detection method was performed. Cells were counterstained with hematoxylin to facilitate cell counting. Twelve randomly chosen fields of each section (72 fields for each group) were counted in a blinded fashion. An apoptotic index was calculated \([100\% \times \text{TUNEL-positive cells}/(\text{total number of cells})]\).

Statistical Analysis.
All data are expressed as mean ± standard error of the mean. Variables measured over time were compared using an analysis of variance for longitudinal data followed by post hoc testing using Bonferroni correction. Other comparisons were tested using one way analysis of variance or student t-test when appropriate. A p value <0.05 was considered significant.
Results

*Effects of MV on pulmonary physiology and hemodynamics.* Arterial oxygen pressures did not differ between the two ventilated groups and remained unchanged throughout the experiment (Figure 1A). After 3 and 4 hrs, PaCO\(_2\) was lower in the HV\(_T\) group, but remained within the normal range (p<0.001) (Figure 1B). Mean arterial pressure and heart rate did not differ between the groups and remained unchanged over time (Figure 1C and 1D). Peak inspiratory pressures were higher in the HV\(_T\) group as compared to the LV\(_T\) group, at 18.7±0.3 vs. 12.8±0.2 cm H\(_2\)O, respectively (Figure 2A).

**Figure 1.** Arterial oxygen pressures, heart rate, means arterial pressures and arterial carbon dioxide levels.

No significant differences in partial pressure of arterial oxygen (PaO\(_2\)), heart rate and mean arterial pressure were observed between the two ventilated groups. After 3 and 4 hrs, PaCO\(_2\) was significantly higher in the LVT group (p<0.001), but values remained within the normal range. Squares: low tidal volume and sepsis, triangles: high tidal volume and sepsis.
Lung injury parameters. No differences in W/D ratio were observed between the controls (5.0 ± 0.1), the sepsis group (4.8 ± 0.1) and the LV T group (5.1 ± 0.1), whereas MV with HV T (5.5 ± 0.1) increased W/D weight ratio as compared to the non-ventilated septic controls (p<0.01). No differences in histological assessment of lungs were observed (data not shown).

Cytokines. Plasma MIP-2, IL-6 and TNF-α levels were measured in healthy control animals, 24 hours after the induction of sepsis, and in septic animals after 4 hrs of MV. No differences were observed between plasma levels of MIP-2, IL-6 and TNF-α among the control, the sepsis and LV T group. MV with high tidal volume (HV T) increased plasma MIP-2 levels compared to the LV T and sepsis groups (p<0.05) (Figure 3).

ICAM-1 and Active PAI-1. ICAM-1 levels in plasma of septic animals were greater than controls (p<0.05; Figure 4A). ICAM levels did not further increase after 4 hr of MV in the septic animals. A similar pattern, although not significant, was observed for ICAM-1 levels in the kidney homogenates. Sepsis increased active-PAI-1 plasma levels but this increase was not significant compared to controls (Figure 4B). MV increased active PAI-1 levels (p<0.01). This increase was greater in the HV T group (Figure 4B). Neither sepsis alone nor sepsis followed by MV resulted in differences in activated PAI-1 levels in kidney homogenates.
**Endothelin-1.** There were no differences in plasma endothelin-1 levels among groups (Figure 4C). In kidney homogenates, sepsis alone significantly increased endothelin-1 levels as compared to controls (p<0.01) and the LV_{T} group (p<0.05).

**Figure 3.** Plasma MIP-2, IL-6 and TNF-α levels.

Plasma MIP-2 levels were higher in the HV_{T} group as compared to LV_{T} or sepsis alone (p<0.05). Plasma IL-6 and TNF-α levels were comparable among groups. LV_{T}: sepsis and mechanical ventilation with low tidal volume, HV_{T}: sepsis and mechanical ventilation with high tidal volume.

**Renal histology and apoptosis.** No differences in histological injury were observed between the groups (data not shown). Sepsis alone did not increase apoptotic index in the renal cortex or the medulla (Figures 5A and C). MV during sepsis was associated with a significantly higher apoptotic index (p<0.01) and this was more significant in the renal medulla. Furthermore, in the renal
medulla we observed a significantly higher apoptotic index in the HV\textsubscript{T} group compared to the LV\textsubscript{T} group (52±10\% vs. 26±7\%, \(p<0.05\)) (Figures 5B and C). In the cortex apoptosis was mainly observed in endothelial cells (Figure 5C).

**Figure 4.** Plasma ICAM-1, PAI-1 and endothelin-1 levels

Plasma soluble intercellular adhesion molecule-1 (ICAM-1) levels increased after sepsis and there was no additional effect of MV (\(p<0.05\)). A similar, although not statistically significant pattern was observed in kidney homogenates ICAM-1 levels (figure 3A). Sepsis and MV increased plasma active plasminogen activator inhibitor-1 (PAI-1) levels as compared to healthy controls. MV with a high tidal volume increased active PAI-1 levels as compared to sepsis alone. MV with a low tidal volume showed a similar, non-significant, trend. No differences in active PAI-1 levels were observed in kidney homogenates (figure 3B). Plasma ET-1 levels did not differ among groups. Sepsis alone increased ET-1 levels in kidney homogenates compared to the other groups (figure 3C). *\(p<0.05\) compared to control, #\(p<0.05\) compared to No MV, $p<0.05$. LV\textsubscript{T}: sepsis and mechanical ventilation with low tidal volume, HV\textsubscript{T}: sepsis and mechanical ventilation with high tidal volume.
Renal function. For technical reasons, we could only continuously monitor urine output during MV. After 4 h of MV, plasma creatinine levels increased in both ventilated groups compared to baseline (p<0.001) (Figure 4). No differences were observed in urine creatinine concentrations. During the final hour, urine production decreased in the HV_T group as compared to the LV_T group.
(0.5 ± 0.1 ml/h vs. 0.9 ± 0.2 ml/h) (p<0.05). In addition, MV with HV caused a roughly 40% decrease in creatinine clearance compared to LV ventilation (p<0.01).

**Figure 6.** Kidney function

No differences were seen in baseline plasma creatinine levels. In both ventilated groups MV increased plasma creatinine levels. Urine creatinine levels did not differ between the two groups. Urine output was lower during ventilation with a high tidal volume. A similar result was observed in creatinine clearance. $p<0.001$, *$p<0.05$, #$p<0.01$. LV: sepsis and mechanical ventilation with low tidal volume, HV: sepsis and mechanical ventilation with high tidal volume.

**Discussion**

The major findings of the present study are: (1) MV with large tidal volumes during sepsis causes apoptosis of kidney cells that was more pronounced in the renal medulla than the renal cortex; this occurred in the absence of overt lung injury or systemic inflammation, and (2) this was associated with a greater than 40% decrease in creatinine clearance compared to LV ventilation during sepsis.
In the current study, we used a double hit animal model with a systemic primary hit and a ventilatory strategy that did not produce gross lung injury. 24h after induction of sepsis, the animals were exposed to 4h of MV using one of two ventilatory strategies that are advocated as safe (6 ml/kg, PEEP 5 cm H₂O) or mildly injurious (15 ml/kg, PEEP 3 cm H₂O). We did not observe histologic injury and peak inspiratory pressures remained stable. The W/D ratio after HV T was only marginally increased but the absolute values are low and are not indicative of lung injury. We also prevented the effects of alterations in PaO₂ and PaCO₂ on renal blood flow by adjusting respiratory rate. Lastly, systemic and renal hemodynamic consequences of different intra-thoracic pressures were avoided by the application of different levels of PEEP, keeping mean airway pressures equal between the ventilated groups.

We found that MV of relatively uninjured lungs led to significant levels of renal apoptosis in both ventilated groups, whereas sepsis alone did not cause apoptosis. Others have shown that sepsis alone may be sufficient to cause apoptosis. Several factors such as timing and puncture size, may account for these differences. The apoptosis was most profound in the HV T group and was associated with decreased renal function compared to LV T as indicated by a decrease in urine flow and creatinine clearance. A recent post mortem study of patients suffering from septic shock induced AKI revealed increased levels of apoptosis. Lerolle et al. performed kidney biopsies within 30 minutes following death in patients with AKI associated with septic shock, and found apoptosis in proximal and distal tubules and occasionally in glomerular cells. In contrast, we observed apoptosis mainly in the medulla, and, to a lesser extent, in the cortex, mainly in endothelial cells. Similar to Lerolle et al, glomerular apoptosis was seldom observed. Apoptosis in septic AKI has not been universally shown. A post-mortem analysis by Hotchkiss et al. showed only few renal abnormalities and no apoptosis using routine microscopy. The pathophysiologic mechanisms leading to apoptosis during septic AKI remain largely unknown.

Apoptotic cell death can proceed by two distinct pathways, the death receptor pathway and the mitochondrial pathway, that ultimately converge to activate effector caspases. The death receptor pathway can be activated by various ligand-death receptor interactions, such as TNF-α-CD120 and Fas Ligand-CD95 interaction. The mitochondrial pathway is activated by several forms of cellular stress such as ischemia and hypoxia, DNA damage and survival factor deprivation. We explored several mechanisms through which activation of apoptosis occurs. These mechanisms correspond to the proposed mechanisms by which MV leads to AKI. Interestingly, we found that the apoptotic index was higher in the medulla than in the cortex.
This is in close concordance with the known regional differences of renal blood flow, with decreased renal blood flow through the medulla as compared to the cortex, suggesting a difference in renal perfusion.\textsuperscript{3} In previous studies we demonstrated the effects of MV on renal blood flow in healthy rats\textsuperscript{30} and the alterations on the microvascular endothelial level during MV of rats treated with intra-tracheal lipopolysaccharide.\textsuperscript{31} In this study we limited effects on renal blood flow by keeping mean airway pressures similar between ventilated groups. However, we cannot exclude an intra-renal shift in blood flow away from the medulla causing regional which could have led to apoptosis mediated via the mitochondrial pathway.

The most common mode of activation of the death receptor pathway is through activation of the TNF-receptor by TNF-α. During biotrauma, whereby MV causes an inflammatory response in the lung, pro-inflammatory mediators, such as TNF-α, may spill-over into the systemic circulation and exert effects on distant organs, including the kidney.\textsuperscript{32,33} This mechanism could possibly explain our results even though plasma levels of IL-6 and TNF-α were unaffected by MV. Plasma MIP-2 levels increased, and even though MIP-2 has not been shown to induce apoptosis in kidney cells,\textsuperscript{12} it may be a sensitive marker of biotrauma, and suggest that other pro-apoptotic molecules which we did not measure may have translocated from the lung into the systemic circulation. A great variety of death-receptors exist, and activation of the death receptor pathway may have occurred via different ligand receptor interactions. For example, previously, Imai et al. showed involvement of Fas Ligand in renal apoptosis during MV after acid aspiration in rabbits.\textsuperscript{34}

The endothelium has been suggested to play an important role in septic organ failure.\textsuperscript{35,36} Our data indicate that renal cortex endothelial cells were particularly susceptible to developing apoptosis. We observed increased serum levels of active PAI-1 after MV in our septic animals. The endothelium is normally a major source of active PAI-1 and increased PAI-1 levels have been associated with a poor prognosis in sepsis.\textsuperscript{37} ET-1 is a potent vasoconstrictor released by the endothelium upon a variety of stimuli in a autocrine/paracrine manner and implicated in renal dysfunction under a variety of circumstances.\textsuperscript{38-42} ICAM-1 is another important marker of endothelial activation. We found increased levels of plasma soluble ICAM-1 during sepsis, independent of MV strategy. Presumably, sepsis alone activated the endothelium without additional effects of MV. A secondary data analysis of 876 patients mechanically ventilated for acute lung injury showed, similar to our results, significantly increased levels of plasma PAI-1, but not ICAM-1 in patients who developed AKI.\textsuperscript{43} The authors suggested that impaired fibrinolysis was important during AKI\textsuperscript{43}, a phenomenon that we, with light-microscopic analysis, could not
confirm. There was no difference in active PAI-1 levels in kidney suggesting a source distant from the kidney as the primary origin of active PAI-1, possibly the lung. In contrast to previous findings in healthy rats, during sepsis ET-1 levels in plasma and kidney homogenates were not affected by MV. Since no endothelial activation, nor systemic inflammation was observed, interventions were therefore not made in this study to definitely define mechanisms by which MV can lead to AKI.

We found that MV with HV may significantly increased renal apoptosis and decreased kidney function. We are not sure if this was a cause-effect relationship, and the exact mechanisms through which renal apoptosis could lead to decreased kidney function remains largely unknown, but this phenomenon has been observed previously. Inhibition of caspases in LPS induced AKI prevented deterioration of renal function. However, inhibition of caspases did not only prevented apoptosis, but also decreased renal inflammation and renal vasoconstriction. Caspase activation and subsequent apoptosis are involved in renal function loss, but likely not through apoptosis alone.

**Conclusions**

In conclusion, we showed that MV during sepsis has profound effects on kidney apoptosis and impairs kidney function. independent of VILI or systemic inflammation. The precise mechanisms by which this occurs are not certain, but do not require severe lung injury. Further research on the link between mechanical ventilation and kidney dysfunction during sepsis are required to mitigate the high mortality in ventilated septic patients.
References


