Energy and Oxygen Metabolism Disorder During Septic Acute Kidney Injury

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Key Words
Septic shock • Acute kidney injury • Apoptosis • Energy metabolism • Renal oxygen metabolism • Renal hemodynamics

Abstract
Background/Aims: Acute kidney injury (AKI) during septic shock, which is one of the most common clinical syndromes in the intensive care unit (ICU), has a high mortality rate and poor prognosis, partly because of a poor understanding of the pathogenesis of renal dysfunction during septic shock. Although ischemic injury of the kidney has been reported to result from adenosine triphosphate (ATP) depletion, increasing evidence has demonstrated that AKI occurs in the absence of renal hypoperfusion and even occurs during normal or increased renal blood flow (RBF); nevertheless, whether energy metabolism disorder is involved in septic AKI and whether it changes according to renal hemodynamics have not been established. Moreover, tubular cell apoptosis, which is closely related to ATP depletion, rather than necrosis, has been shown to be the major form of cell injury during AKI. Methods: We used canine endotoxin shock models to investigate the hemodynamics, renal energy metabolism, renal oxygen metabolism, and pathological changes during septic AKI and to explore the underlying mechanisms of septic AKI. Results: The present results revealed that the nicotinamide adenine dinucleotide (NAD+1) pool and the ATP/adenosine diphosphate (ADP) ratio were significantly decreased during the early phase of septic AKI, which is accompanied by a decreased renal oxygen extraction ratio (O2 ER%) and decreased renal oxygen consumption (VO2). Furthermore, significant apoptosis was observed following renal dysfunction. RBF and renal oxygen delivery were not significantly altered. Conclusion: These results suggest that imbalanced energy metabolism, rather than tubular cell apoptosis, may be the initiator of renal dysfunction during septic shock.

Rong-li Yang and Xiao-ting Wang contributed equally to this work and thus share first authorship.

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Introduction

Sepsis or septic shock is notably the most predominant cause of acute kidney injury (AKI) in critically ill patients [1, 2]; however, the pathogenesis of renal dysfunction during septic shock has not been established. Currently, the hypotheses based on clinical and animal experiments are primarily focused on renal hemodynamics, inflammatory responses, sympathetic nerve activity, and renal tubular cell death [3, 4]. Whether energy and oxygen metabolism disorders are involved in the complicated start of renal injury and whether they are correlated with renal hemodynamics and tubular cell death have not been established.

It appears that hyperdynamic circulation occurs most frequently in septic shock patients during the early phase. In the majority of these patients, RBF either remains the same or is increased (3). Additionally, various views concerning changes in renal metabolism (including oxygen delivery, extraction, and consumption), tubular reabsorption, and glomerular filtration rate (GFR) [2, 5] tend to yield contradictory underlying mechanisms of septic AKI. A new concept that microcirculatory dysfunction, rather than hypoperfusion, is a major contributor to the pathogenesis of AKI [6, 7], which has not been supported by credible evidence, raises the question of what is the real cause of renal injury during septic shock.

Recently, apoptosis has been reported to be the major form of renal cell injury [4, 8, 9]; however, we question whether the number of apoptotic cells observed in the kidney is large enough to be significant because we possess two kidneys with compensatory ability. Furthermore, most injured tubular cells progress toward apoptosis rather than necrosis, thus leading us to believe that whatever causes renal cell apoptosis may be responsible for early renal dysfunction. Several publications have reported that mitochondrial damage and energy metabolism disorder are characteristics of apoptosis, and the cellular ATP level may represent a threshold dividing apoptosis from necrocytosis [10-12]. Moreover, the rapid recovery of the ATP level can inhibit cell apoptosis and minimize organ dysfunction [13]. Based on this evidence, we can conclude that energy metabolism plays a significant role in cell apoptosis. If the dysfunction of renal microcirculation is critical in the pathogenesis of septic AKI, then energy and oxygen metabolism disorders should be observed in the early phase of renal dysfunction. Guided by this evidence, our study assessed the dynamic changes of RBF, renal function, renal oxygen metabolism, renal energy metabolism, and cell death patterns to analyze what type of role energy metabolism and cell apoptosis play in septic AKI pathogenesis and whether they are accompanied by oxygen metabolism disorder.

Materials and Methods

Animal preparation

All experimental and surgical procedures were approved by the Institute of Animal Care and Use Committee, and all efforts were made to minimize the animals’ suffering and the number of canines involved in the experiment. Except for animals that died before monitoring was performed, 24 mongrel canines (19.2 ± 2.1 kg) were raised in a quiet and warm environment according to the Principles of Laboratory Animal Care, and they had no access to food but free access to water for 12 h before the experiment. The canines were randomly divided into control (n=6), 2-h (n=6), 4-h (n=6), and 6-h (n=6) groups, before which the baseline parameters, including gender, weight, and height, were analyzed to avoid nonspecific differences.

Chemicals and reagents

Lipopolysaccharide (LPS) (∵Escherichia coli, O55:B5), ATP, ADP, NAD⁺, and phosphocreatine (PCr) were purchased from Sigma-Aldrich Corp. Pentobarbital sodium was obtained from Merck Corp. The In Situ Cell Death Detection Kit was purchased from Roche Applied Science.
Model of LPS-induced septic AKI

The canines were anesthetized by an intraperitoneal injection of pentobarbital (30 mg/kg) and placed in the supine position. Tracheal intubation was performed, and the endotracheal tube was connected to a Servo Ventilator 900C breathing machine (VT=8-10 ml/kg, f=15-20/min, PEEP=0 mmHg, FiO₂=30-50%, PetCO₂=25-35 mmHg) to maintain the PaO₂ at approximately 100 mmHg. A dual-lumen catheter (Edwards) was inserted into the right femoral vein, which allowed for continuous pumping of liquid, anesthetics (pentobarbital, 4 mg/kg/h), and neuromuscular blocking agents (NMBAs, vecuronium at an initial dose of 0.1 mg/kg, followed by 0.05 mg/kg/h). A PICCO arterial duct and Swan-Ganz (Edwards) catheter were inserted into the right femoral artery and right external jugular vein, respectively, for continuous recording of systemic hemodynamic values and blood sampling. The RBF was measured through the left renal artery using a Doppler flow meter (TS410, Transonic Systems, Ithaca, NY, USA). Controlled infusion of LPS (2 mg/kg, at 1 mg/kg·min) was conducted through a pulmonary artery catheter 30 min postoperatively when the vital signs were stable [14]. Normal saline (4.3±0.6 ml/kg·h) was instilled to maintain the central nervous pressure (CVP) and pulmonary artery wedge pressure (PAWP) at baseline levels to prevent hypodynamic circulation; it was important to simulate the most common clinical scenario in patients. Samples of the cortex, corticomedullary zone, and medulla were separately harvested 0, 2, 4, and 6 h after LPS infusion in the control, 2-h, 4-h, and 6-h groups.

Hemodynamic monitoring

Systemic hemodynamic indexes, such as mean arterial pressure (MAP), systolic arterial pressure (SAP), and stroke volume (SV), were continuously recorded using PICCO, whereas PAWP and PVR were monitored using a Swan-Ganz (Edwards) catheter. Blood samples from the kidney were collected from the Swan-Ganz catheter (the blood gas values in the femoral artery blood and renal artery blood were the same), and a trocar was inserted into the left renal vein. RBF was measured using Doppler flow probes (PS & PR Series, Transonic Systems). These data were collected and stored using the Data Acquisition System at 1-minute intervals throughout the experiment.

Systemic and renal metabolism

Blood samples were collected using PICCO and the Swan-Ganz catheter to analyze oxyhemoglobin saturation (SO₂), oxygen pressure (PO₂), partial pressure of carbon dioxide (PCO₂), pH, base excess (BE), electrolytes, and renal function at 1-hour intervals. Systemic and renal oxygen metabolism indexes, such as oxygen delivery (DO₂), VO₂, and O₂ER, were measured using a blood gas analyzer (Radiometer ABL 520, Denmark). A urinary catheter was inserted into the bladder to monitor the urine output. The filtration fraction (FF) was calculated as the creatinine clearance rate (CCr)/RBF*(1-Hct). Finally, the level of lactate in the arterial blood was measured as a sensitive biomarker of septic AKI.

Hematoxylin-eosin staining for renal pathophysiology

Renal tissues were immediately harvested and fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned at a 4-μm thickness 2, 4, or 6 h after LPS infusion in the experimental group and 6 h after saline infusion in the control group. The sections were subsequently analyzed by hematoxylin-eosin (HE) staining [15] and mounted using neutral balsam. The renal cortex, corticomedullary zone, medulla layer, and inner medulla were primarily observed using Olympus IX51 optical microscopy. Semiquantitative scores for tubular cell injury and acute tubular cell necrosis (ATN score) were used to evaluate renal damage by histology. The percentage of tubules that represented cellular necrosis, loss of brush border, vacuolization, cast formation, and tubule dilation was scored as follows: 0 (none) (1-10%), 2 (11-25%), 3 (26-45%), 4 (46–75%), and 5 (>76%).

TUNEL for apoptosis

In situ renal cell apoptosis was detected by the TdT-mediated dUTP nick-end labeling (TUNEL) assay, in accordance with the detection kit protocols. The renal tissues were embedded in paraffin, fixed with paraformaldehyde, and cut at a 5-μm thickness. For preciseness, the observers were blinded to the groups. The slices were dewaxed at 60°C overnight, washed with dimethylbenzene, and subsequently hydrated using 100%, 95%, 90%, 80%, and 70% alcohol. After hydration, the sections were digested with protein K (Protein K 20 µg/ml in 0.1 mol/l Tris-HCL, pH7.4) at 21-37°C for 15 min, inactivated by 2% hydrogen...
peroxide for 5 min at room temperature, washed with phosphate-buffered saline (PBS) twice, and labeled with TUNEL reaction mixture for 60 min at 37°C in the dark. Apoptosis was assessed using a computer-assisted Olympus IX51 fluorescence microscope, and the number of TUNEL-positive cells was calculated by observers who were blinded to each group. The cells in the renal cortex, corticomedullary zone, medulla layer; and inner medulla were primarily counted in 3 randomly selected fields.

**HPLC to assess energy metabolism**

High-performance liquid chromatography (HPLC)\(^1\)\(^2\),\(^1\)\(^7\) was conducted to measure the concentrations of energy substances, including ATP, ADP, NAD\(^+\), and PCr, in different portions of the kidney. Numerous preliminary tests were performed to avoid degeneration of nucleotides. Renal tissues from different groups were preserved in liquid nitrogen within a few seconds after sampling. The frozen renal tissue (200 mg) was mixed with 0.42 M perchloric acid (cold solution), ground for 10 min, and centrifuged (4°C) at 6000\(\text{g}\) for 10 min. The supernatant (1 ml) was neutralized with 0.5 mol/l KOH until the pH was 7.0, and the mixture was subsequently replenished to 2 ml. After standing for 10 min, the mixture was centrifuged (4°C) at 6000\(\text{g}\) for 10 min, and the supernatant was filtered using a 0.45-μm sieve to eliminate the impurities. Finally, 20 μl of filtrate of each sample was injected directly into the reverse-phase HPLC system (Waters Alliance, USA) with a column (Waters Nova-Pak C18) temperature of 20-25°C. The mobile phase was composed of 220 mmol/l KH\(_2\)PO\(_4\)/K\(_2\)HPO\(_4\) buffer (pH 7.0), 3 mmol/l tetrabutylammonium hydroxide, and 5% carbinol; the flow rate was 0.9 ml/min. The absorbance of ATP, ADP, and NAD\(^+\) was measured at 254 nm and that of PCr was measured at 210 nm. The final concentration of energy substances from each sample was calculated according to the standard curve obtained from standard samples.

**Statistical analysis**

All of the data were analyzed using the SPSS 13.0 statistical analysis package. An intergroup comparison of measurement data was performed using an independent-samples t-test, and a nonparametric test was used if equal variance was not assumed. The results are presented as the mean and standard deviation (mean±SD) values. \(P<0.05\) was considered to be statistically significant, and \(P<0.01\) was considered to indicate remarkable significance.

**Results**

**Systemic and renal hemodynamics**

Systemic hemodynamic indexes showed a non-significant difference at baseline (1 h) between groups (\(P>0.05\)) (Fig. 1A-I). After LPS infusion, the MAP (Fig. 1B) decreased (from 147.0±10.7 mmHg at 0 h to 90.0±17.3 mmHg at 1 h; \(P=0.0102\)) compared with baseline levels and the control group, and it only showed a non-significant increase after saline administration, which successfully maintained the CVP (Fig. 1C) and PAWP (Fig. 1E) at baseline levels; a similar decrease was also observed in SVR (Fig. 1D) from 1 to 5 h (from 3.62±1.02 l/min at 0 h to 3.08±0.98 l/min at 1 h; \(P=0.0014\)). Additionally, EVLW (Fig. 1G) only showed a significant increase at 6 h (\(P=0.014\)), whereas non-significant differences were observed in other systemic hemodynamic indexes, including CO (Fig. 1A) and GEDV (Fig. 1F). For renal hemodynamics, RBF (Fig. 1H) and renal vascular resistance (RVR) (Fig. 1I) were measured. After LPS infusion, RBF showed a non-significant increase compared with baseline levels and the control group (\(P=0.72\)), whereas RVR decreased prominently from 1 to 3 h (\(P=0.028\)).

**Systemic and renal oxygen metabolism**

Systemic oxygen metabolism, including \(\text{DO}_2\) (Fig. 2A), \(\text{VO}_2\) (Fig. 2B), and \(\text{O}_2\text{ER}\) (Fig. 2C), exhibited non-significant changes after LPS infusion compared with the control group (\(P>0.05\)). Renal \(\text{VO}_2\) (4.87±1.58 ml/min at 4 h in the control group vs. 2.47±0.95 ml/min at 4 h in the LPS groups; \(P=0.0026\)) (Fig. 2E) and \(\text{O}_2\text{ER}\) (17.29±2.36% at 2 h in the control group vs. 8.02±1.35 ml/min at 2 h in the LPS groups; \(P=0.0012\)) (Fig. 2F) decreased significantly, whereas renal \(\text{DO}_2\) (Fig. 2D) showed non-significant change.
Fig. 1. Systemic and renal hemodynamics. LSP infusion (yellow) induced significant decreases in MAP, SVR, and RVR compared with the control group (green), together with an increase of EVLW, whereas CO, GEDV, and RBF remained almost unchanged. Saline infusion successfully maintained CVP and PAWP at baseline levels. *Significant difference in the LPS groups compared with the control group (P<0.05); **prominent significant difference (P<0.01). KC, cortex of the kidney; KI, boundary of the cortex and medulla; KM, medulla of the kidney.

Fig. 2. Systemic and renal oxygen metabolism. LSP infusion (yellow) induced significant decreases in renal VO_2 and renal O_2ER compared with the control group (green), whereas systemic DO_2, VO_2, O_2ER, and renal
Renal function and septic shock index

The serum creatinine (SCr) (Fig. 3A) showed a slow increase from 67.00±9.51 μM/l (0 h) to 98.03±18.88 μM/l (6 h) compared with the baseline levels and the control group (P=0.015). Moreover, CCr (Fig. 3C) prominently decreased from 101.16±19.28 ml/min (at 0 h) to 29.23±15.69 ml/min (at 6 h) (P=0.0023), whereas the osmotic pressure exhibited a significant decrease from 1081.17±189.48 mOsm/kg·H$_2$O (at 0 h) to 287.50±109.94 mOsm/kg·H$_2$O (at 6 h) (P=0.00103). Urinary NAG (Fig. 3G) showed a significant increase in a time-dependent manner after LPS infusion. Urine creatinine (UCr) (Fig. 3B) decreased compared with the baseline and control group values (P<0.05). Additionally, the fractional excretion of sodium (FENa) (Fig. 3E) decreased significantly at 4 h after LPS infusion (P=0.015). Urinary output (Fig. 3H) showed a significantly lower level in the LPS 2-h group compared with that at the same time point in the control group (P<0.01), followed by a slow increase.
2 h afterward. Lactate (Fig. 3I), which is a biomarker of septic shock, showed a significant increase 2 h after LPS infusion.

Renal tubular cell injury demonstrated by HE staining

An intact and clear renal tissue structure was observed in the control group (Fig. 4 A,E,I), whereas the LPS groups showed significant swelling, vacuolar degeneration, and cellular edema, primarily in the renal cortex (Fig. 4B-D) and corticomedullary zone (Fig. 4F-H), which appeared to gradually worsen in a time-dependent manner. Non-obvious necrosis was observed throughout the entire kidney. The tubular basement membrane, collecting tubule, and glomerulus in the LPS group were not different from the control group. The number of apoptotic cells only showed a significant increase 6 h after LPS infusion in the renal medulla (Fig. 4J-L).

Tubular cell apoptosis assessed by TUNEL staining

To explore whether LPS infusion caused cell apoptosis in the kidney, TUNEL staining was performed in each group. The results showed that TUNEL + cells were primarily observed in the proximal renal tubule in the cortex (Fig. 5A-D) and at the boundary of the cortex and medulla (Fig. 5F-H). The distribution of these cells was similar to that observed in tubular...
cell injury as assessed by HE staining. The number of apoptotic cells significantly increased (3±1/mm² in the control group to 2351±682/mm² in the 6-h group, P<0.01) in a time-dependent manner in the LPS groups compared with the control group (Fig. 5M).

**Discussion**

Whether apoptosis or necrosis is the primary type of injury in septic AKI has been the focus of a polarized argument because of their decisively different therapeutic methods. Recent studies have reported that only slight tubular cell necrosis and significant apoptosis are the primary pathological changes associated with septic AKI [18, 19]. However, the underlying mechanisms of tubular cell apoptosis and renal dysfunction have not been
established. Moreover, the number of apoptotic tubular cells reported in the present study and other studies may not be large enough to induce severe renal injury or renal failure, thus stimulating our curiosity concerning what the actual determinant of renal dysfunction is in the early phase of septic AKI. The present study aimed to assess the hemodynamic changes in oxygen and energy metabolism in the early phase of septic AKI to explore whether energy metabolism is associated with renal dysfunction and tubular cell apoptosis.

The present study demonstrated that after LPS injection, canines presented significant renal dysfunction, indicated by significantly decreased CCr, FF, urinary Na, and urinary osmosis, as well as significantly increased SCr and urinary n-acetyl-beta-D-amino glycosidase enzymes (NAG), in the early phase (2 h). HPLC analysis revealed apparent decreases in the ATP/ADP ratio and NAD$^+$ level (2 h), which was followed by a non-significant increase 6 h afterward. Additionally, HE and TUNEL staining indicated significant tubular cell injury and apoptosis 4 h after LPS infusion, which indicated that renal dysfunction occurred before tubular cell apoptosis. Furthermore, the number of apoptotic cells was not large enough to trigger renal dysfunction because a patient may suffer from severe kidney trauma without renal dysfunction. In summary, these factors suggest that apoptosis may not be the initiator of renal dysfunction and that functional energy impairment is more likely to be responsible for renal dysfunction.

According to the literature, the depletion of ATP can induce apoptosis, which can exacerbate cell injury and organ dysfunction [20, 21]. In energy metabolism disorders, energy-requiring biochemical processes enter into a perturbed state and subsequently cause a series of characteristic morphological changes, such as plasma membrane blebbing, which distinguishes apoptosis from necrosis (18). The present study showed a marked decrease in the ATP level and ATP/ADP ratio, primarily in the renal cortex of the kidney, before apoptosis was observed. Additionally, energy disorder and tubular cell apoptosis presented similar distributions, primarily in the renal cortex, which indicated that ATP depletion may be correlated with tubular cell apoptosis in the canine model of septic AKI. Except for ATP depletion, the present study showed a significantly decreased NAD$^+$ level, which was followed by a slow recovery. Once DNA has been damaged, NAD$^+$ can be dissociated by PARP into ADP-ribose monomers and NAD, leading to extensive consumption of NAD$. Consequently, a large amount of ATP is needed to restore the level of NAD$, and cells will finally head toward a specific programmed cell death pathway involving NAD$^+$/ATP depletion [22-24], which suggests that NAD$^+$ depletion may be one of the reasons for the observed ATP decrease and renal dysfunction in our study.
Several recent studies have suggested that although RBF was not significantly reduced, a decline in renal microcirculation was detected in the renal cortex, even in the non-hypoxic area [25, 26], which could lead to renal hypoxia. Consistent with other studies [2, 25], RBF and renal DO$_2$ showed non-significant decreases in the present study, whereas renal VO$_2$ and O$_2$ER decreased, indicating that a renal microcirculatory disturbance or cellular failure, such as mitochondrial dysfunction, may be associated with energy disorder, cell apoptosis, and renal dysfunction in the AKI model. Recent studies have maintained that microcirculatory dysfunction in the renal cortex or renal medulla can occur despite normal or increased RBF [27]. The microcirculation is recognized to play an important role in delivering and regulating the exchange of oxygen in the surrounding tissues, which is critical for energy metabolism and cellular function [28]. This evidence indicates that microcirculatory dysfunction may be the cause of renal VO$_2$ and O$_2$ER decreases and the subsequent energy metabolism disorder. However, additional studies are warranted to explain why VO$_2$ is stabilized thereafter. Is there any correlation between energy metabolism and mitochondrial dysfunction? Other studies have indicated that PARP can lead to NAD$^+$ depletion and mitochondrial release of apoptosis-inducing factors, which are key mediators of cell death [29]. A recent study demonstrated that NO and ROS triggered PARP-independent cell death and mitochondrial dysfunction; however, PARP inhibition did not modify the loss of mitochondrial function [30]. Another study demonstrated that energy repletion with NAD or a NAD/pyruvate mixture inhibited mitochondrial swelling and microtubule depolymerization [31]. These results suggest that many studies support a close relationship between NAD loss and mitochondrial dysfunction [29, 32], which may also be the cause of decreased renal VO$_2$. However, the evidence from the present study was insufficient to reveal mitochondrial failure and the causal relationship between NAD$^+$ depletion and mitochondrial dysfunction; thus, additional studies are needed.

In the present study, energy metabolism disorder and tubular cell injury were primarily observed in the renal cortex, whereas only non-significant damage was detected in the medulla. The reason for these observations was not clear, although they may be related to differences between species, blood flow, and experimental conditions. An earlier study reported a similar distribution and showed that the basal interstitial concentration of adenosine in the renal cortex was higher than that in the medulla [33]. Additional studies must be conducted to determine whether this finding can be duplicated and confirmed in other animals and to validate the key mechanism.

**Conclusion**

The present study indicated that tubular cell apoptosis may not be the cause of renal dysfunction during septic AKI and that ATP depletion, which may originate from NAD$^+$ depletion caused by DNA damage, is more likely to be the cause of renal dysfunction or even tubular cell death. However, because of the very short time period of the septic AKI study, we recommend that longer and more experimental and clinical studies be performed. Moreover, many studies have shown that NAD$^+$ administration can alleviate oxidative cell death, trauma, and X-ray-induced tissue injury. Additional experiments will explore whether NAD$^+$ metabolism disorder plays a key role in renal dysfunction and whether NAD$^+$ administration can become a therapeutic method for preventing and treating septic AKI.

**Disclosure Statement**

Rong-li Yang and the other co-authors declare that they have no conflicts of interest.
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References