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EARLY FLUID PLUS NOREPINEPHRINE RESUSCITATION DIMINISHES KIDNEY HYPOPERFUSION AND INFLAMMATION IN SEPTIC NEWBORN PIGS

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Abstract

Sepsis is the most frequent risk factor for acute kidney injury (AKI) in critically ill infants. Sepsis-induced dysregulation of kidney microcirculation in newborns is unresolved. The objective of this study was to use the translational swine model to evaluate changes in kidney function during the early phase of sepsis in newborns and the impact of fluid plus norepinephrine resuscitation. Newborn pigs (3–7-day-old) were allocated randomly to three groups: 1) sham, 2) sepsis (cecal ligation and puncture) without subsequent resuscitation, and 3) sepsis with lactated Ringer plus norepinephrine resuscitation. All animals underwent standard anesthesia and mechanical ventilation. Cardiac output and glomerular filtration rate were measured noninvasively. Mean arterial pressure, total renal blood flow, cortical perfusion, medullary perfusion, and medullary tissue oxygen tension ($mtPO_2$) were determined for 12 h. Cecal ligation and puncture decreased mean arterial pressure and cardiac output by more than 50%, with a proportional increase in renal vascular resistance and a 60–80% reduction in renal blood flow, cortical perfusion, medullary perfusion, and $mtPO_2$ compared to sham. Cecal ligation and puncture also decreased

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glomerular filtration rate by ~79% and increased AKI biomarkers. Isolated foci of tubular necrosis were observed in the septic piglets. Except for $mtPO_2$, changes in all these parameters were ameliorated in resuscitated piglets. Resuscitation also attenuated sepsis-induced increases in the levels of plasma C-reactive protein, proinflammatory cytokines, lactate dehydrogenase, alanine transaminase, aspartate aminotransferase, and renal NLRP3 inflammasome. These data suggest that newborn pigs subjected to cecal ligation and puncture develop hypodynamic septic AKI. Early implementation of resuscitation lessens the degree of inflammation, AKI, and liver injury.

Keywords

Newborn sepsis; acute kidney injury; renal hemodynamics; pig model; fluid and norepinephrine resuscitation

BACKGROUND

Sepsis is an overwhelming host response to infection that results in devastating and life-threatening organ dysfunction, frequently comprising acute kidney injury (AKI) (1). Forty-nine million people are estimated to be affected by sepsis yearly, and more than 11 million deaths result from this syndrome (2). Sepsis is also the leading cause of death in critically ill infants globally and the most frequent risk factor for AKI in this population, accounting for approximately 45–70% of cases of AKI in pediatric intensive care units (1,3). The development of AKI increases mortality by about 30% in pediatric septic patients (4). Newborns are at extreme risk of severe septic infection because of their immature immune systems. Therefore, infants with sepsis-induced AKI come down with worse outcomes, such as lengthy hospital admissions, higher mortality rates, reduced quality of life, and risk of long-term disability (4–6).

Septic AKI is characterized by reduced kidney function that manifests with elevated plasma creatinine and blood urea nitrogen, reduced glomerular filtration rate (GFR), and urine output within 7 days of sepsis onset (7–9). Septic AKI can be categorized as early or late. Early septic AKI develops within 48 h of sepsis, while AKI occurring between 48 h and 7 days of sepsis onset is late septic AKI (10). The pathophysiology of this disease is poorly understood, especially in infants. Sepsis-induced AKI is a complex disorder that occurs because of infections, host response to infection, or indirect mechanistic processes caused by the consequences of sepsis or sepsis therapy (11). Multiple mechanisms such as macrocirculatory dysregulation and microcirculatory dysfunction, systemic and renal inflammation, cellular metabolic reprogramming, mitochondrial dysfunction, hypoxia, oxidative stress, and renin-angiotensin-aldosterone system dysfunction have been implicated in the pathogenesis of septic AKI. These diverse pathophysiological mechanisms involved in septic AKI make the development of specific effective therapy for the treatment or prevention of this disorder challenging (10).

Newborns possess several distinct physiological features pre-disposing to septic AKI (12). Renal vascular resistance is elevated in newborns. The neonatal kidney only receives 5–10% of cardiac output (COP), resulting in reduced total renal blood flow (RBF) compared to 20–25% in adults (12,13). Newborns have limited autoregulatory capacity to control the effects

of mean arterial pressure (MAP) fluctuations on kidney functions (14). Moreover, the unique immune phenotype in early life (15,16) and immature myocardial function (17) increase newborn susceptibility to severe septic shock with multiorgan dysfunction (15,18). These factors indicate that the pathophysiologic mechanisms and disease phenotype of neonatal septic AKI may differ from adults (19).

There are very few studies on sepsis-induced AKI in the pediatric population compared to the abundant literature on adult sepsis and septic AKI. Altered renal macrocirculation and hypoperfusion were previously considered to be the main mechanisms of septic AKI. Still, many experimental and clinical studies have demonstrated that AKI develops in sepsis regardless of preserved or elevated RBF (20). These findings suggest that the intra-renal mechanisms driving this disorder are yet to be resolved (20). The role of renal microcirculatory dysregulation resulting in renal tissue hypoperfusion and hypoxia in the pathogenesis of septic AKI has not been fully elucidated, especially in newborns (21,22).

The current interventions for neonatal septic AKI involve antimicrobial therapy, vasopressor therapy, volume resuscitation, and renal replacement therapy. They are mostly focused on mechanisms upstream from the kidney or, at best, supportive strategies to keep the patients alive, hoping the kidney function will eventually recover (23,24). Therefore, uncovering novel therapeutic approaches for managing sepsis-induced AKI that will ensure the transition from supportive treatment strategies is pertinent, but this is dependent on the understanding of the complicated pathophysiological mechanisms of this disease in newborns (23). Most experimental studies on septic AKI use adult animal models; hence, translating their findings to newborns may not produce the most appropriate therapeutic or pharmacological effects due to the immaturity of the cardiovascular system and immunological responses in newborns (15,18).

Early detection and treatment of sepsis-related dysregulation of tissue perfusion are critical to reducing mortality in septic shock patients (24). Early fluid and vasopressor resuscitation is the standard of care for sepsis patients to ensure recovery from hypotension and sustain adequate tissue perfusion and oxygen delivery (23). Previous studies have revealed that early norepinephrine (NE) treatment in hypotensive septic patients increases COP and improves microcirculation (25). However, patients may also become unresponsive to NE and develop refractory hypotension and septic shock, with prolonged and high doses of NE associated with increased mortality and impaired organ function (26).

Early identification, management, and suitable resuscitation are crucial for improving survival rates in infants with septic AKI. However, few studies have investigated the role of volume resuscitation and vasopressor therapy in newborns. Unlike swine, rodents are poor models of human infant kidney pathophysiology. In this study, we use the translational swine model to evaluate the changes in renal hemodynamics during the early phase of sepsis in 3- to 7-day-old piglets. We also examined the impact of early fluid plus NE resuscitation on renal hemodynamics in the setting of newborn septic AKI.

MATERIALS AND METHODS

Animals

All procedures followed national standards for the care and use of laboratory animals. The Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center, Memphis, reviewed and approved the protocol (# 21-0276). Male newborn pigs (3–7 days old) obtained from Nichols Hog Farm (Olive Branch, MS) were allocated randomly to three groups: 1) sham with no cecal manipulation (sham); 2) cecal ligation and puncture (CLP)-induced sepsis without subsequent resuscitation (CLP), (3) CLP-resuscitated sepsis with early fluid and NE administration (CLP + RST). The size of our piglet groups was established based on studies with the same porcine model of neonatal sepsis by us and other labs (27–29).

Anesthesia and mechanical ventilation

The newborn pigs were anesthetized using a mixture of 20 mg/kg of ketamine (100 mg/mL, Covetrus, Dublin, OH) and 2.2 mg/kg of Xylazine (100 mg/mL, Covetrus, Dublin, OH), administered intramuscularly into the semimembranosus muscle of the rear leg. Subsequently, the piglets were placed in a supine position on a heating pad, and the depth of anesthesia was confirmed before performing a tracheotomy using a 3.5-mm endotracheal tube. Pressure-controlled mechanical ventilation (Sechrist Millennium® neonatal/infant ventilator, Anaheim, CA) was established using the following settings: fraction of inspired oxygen 0.21, peak inspiratory pressure 15 cmH_2O, positive end-expiratory pressure 5 cmH_2O, respiratory rate 20 rpm, and inspiratory time 0.6 s. The femoral vein was catheterized for fluid and medication administration. Induction and maintenance of general anesthesia were achieved *via* intravenous (IV) α-chloralose, 50 mg/kg loading dose, followed by 20 mg/kg intermittent boluses. Arterial blood gas, pH, and hematocrit were monitored periodically throughout the experiment with a GEM Premier 3000 Blood Gas Analyzer (Instrumentation Laboratory, Bedford, MA). Ventilator settings were adjusted to maintain PCO_2, PO_2, and pH at physiological ~30 mm Hg, >85 mm Hg, and 7.4, respectively. Animals were monitored repeatedly during experiments for anesthesia depth and redosed as needed. Urine was drained *via* a ureteral catheter.

Renal and systemic hemodynamics measurement

Cardiac output was measured noninvasively using electrical velocimetry (Aesculon Electrical Velocimetry, Osypka Medical GmbH) (30). Mean arterial pressure was measured *via* 3.5 Fr intra-arterial pressure catheters inserted in the right femoral artery (Mikro-Tip SPR-524, Millar Instruments, Inc, Houston, TX) connected to a physiological pressure transducer (ADInstruments, Colorado Springs, CO). Piglets were then placed in the right lateral semirecumbent position to measure renal hemodynamics. The left kidney was exposed retroperitoneally through flank incisions to permit access and dissect the renal pedicle. Renal blood flow was measured using a transit-time ultrasound perivascular flow probe (Transonic 2 mm PS, Ithaca, NY) placed around the main renal artery and connected to a flowmeter (Transonic Systems). Local cortical (coPf) and medullary (mePf) perfusion were recorded using laser Doppler flowmetry (Periflux 5000, Perimed Inc). In addition, a fiber-optic probe (OxyLite Pro, Oxford Optronix) was advanced to measure medullary tissue

oxygen partial pressure ($mtPO_2$). All recordings were acquired simultaneously using the PowerLab data acquisition system (ADInstruments, Colorado Springs, CO) and LabChart Pro software. Baseline (0 h) hemodynamics were obtained once all monitoring devices were in place, and data was recorded continuously throughout the experiment.

Polymicrobial sepsis induction

Sepsis was induced in anesthetized and mechanically ventilated neonatal pigs with the CLP technique. A left paramedian incision was performed to identify and expose the cecum and terminal ileum. The cecum was ligated distal to the ileocecal junction and punctured 7 times using an 18-gauge needle. Fecal matter was then extruded from the punctured cecum into the peritoneum. The cecum was returned to the abdominal cavity, which was sutured in layers, starting with the peritoneum. For sham-operated piglets, the abdominal wall was closed after identifying the cecum without any further manipulation. A detailed description of the surgical procedure has been published in our prior study (31). The newborn pigs were maintained under anesthesia and mechanical ventilation for 12 h after the surgical procedure.

Resuscitation protocol

All piglets were given maintenance IV fluid using lactate Ringer at 4 mL/kg/h. Resuscitation was initiated at 4 h after CLP in the CLP-resuscitated group. An IV fluid bolus of up to 60 mL/kg was given, followed by NE continuous IV infusion initiated at 0.05 ug/kg/min and titrated up to 0.8 ug/kg/min. The goal of the resuscitation protocol was to maintain MAP within 15 mmHg from baseline.

Sample collection

Twelve hours after CLP, blood was collected from the femoral artery catheter into heparin tubes. The tubes were subsequently centrifuged, and plasma was collected and frozen for analysis. Urine samples were centrifuged and analyzed or stored frozen. Newborn pigs were euthanized with 0.2 mL/kg Euthasol (20% sodium pentobarbital and phenytoin sodium). The kidneys were harvested, sectioned transversely, and stored in 4% buffered formalin for histopathological analysis or frozen in a freezer (−80°C).

Kidney injury and inflammation assay

Kidney function in the newborn pigs was evaluated by measuring GFR, plasma creatinine concentration, and blood urea nitrogen (BUN). Liquid chromatography-tandem mass spectrometry was used to determine creatinine concentrations at the UAB/UCSD O'Brien Core Center for AKI Research at the University of Alabama at Birmingham. Blood urea nitrogen level was measured using a colorimetric detection kit K024-H1 (Arbor Assays, Ann Arbor, MI). Neutrophil gelatinase-associated lipocalin (NGAL) was measured in the plasma using the pig NGAL enzyme-linked immunosorbent assay (ELISA) kit (BioPorto Diagnostics, Hellerup Denmark). Plasma C-reactive protein (CRP) was determined using an ELISA kit (Eagle Biosciences, Amherst, NH). Multiple proinflammatory cytokines were simultaneously measured in plasma with multiplex technology using the Luminex MAGPIX system (DiaSorin Inc, Saluggia, Italy). The porcine multiplex kit was purchased from

Millipore Sigma (St. Louis, MO). Liver injury was evaluated by measuring plasma alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH), which were determined using the DiaSys Respons910VET Chemistry System analyzer (Holzheim, Germany).

GFR determination

The three-compartment method with fluorescein-isothiocyanate (FITC) conjugated sinistrin clearance was used to determine GFR, as we previously described and validated in piglets (31). The method is based on the clearance of FITC-sinistrin as assessed by a fluorescence detector. Briefly, while the animals were under anesthesia, a predetermined region on the flank was shaved and cleaned. The optical transdermal GFR device (MediBeacon GmbH, Mannheim, Germany) was prepared, and once the battery was connected, the device was attached with an adhesive patch to the skin surface of the piglet. After 3–5 min of steady background reading, FITC-sinistrin solution (50 mg/mL) was administered intravenously in a smooth and rapid bolus at 20 mg/Kg b. wt. The device recorded FITC-sinistrin clearance for 4 h. The device was then removed, and the data were analyzed with the MediBeacon Studio 3 software following the manufacturer's instructions.

Histopathology

Histopathological analysis was independently performed by Probetex (San Antonio, TX). Kidney samples were fixed in 4% neutral-buffered formalin, dehydrated in graded alcohols, and embedded in paraffin. The samples were then sectioned for hematoxylin and eosin staining and examined by a pathologist at Probetex in a blinded manner. Piglets subjected to CLP showed minimal tubular injury in the form of coagulative necrosis, casts, or dilatation. Thus, the slides were only graded for tubular necrosis using a scale of 0–5 where 0 = normal, 1 = minimal affecting 1 to 10% of cortex, 2 = mild and multifocal affecting 11 to 25% of cortex, 3 = moderate and multifocal affecting 26 to 50% of cortex, 4 = marked affecting 51 to 75% of cortex, and 5 = severe and diffuse affecting greater than 75% of cortex.

Immunofluorescence

Kidney slices were fixed in 4% formaldehyde for about 20 min, followed by washing with phosphate-buffered saline and permeabilized with 0.1% Triton X-100 for about 15 min. The samples were incubated in 2% BSA to block nonspecific binding sites and treated overnight at 4°C with an NLRP3 primary antibody at 1:100 dilution. The next day, the samples were washed with phosphate-buffered saline and incubated with the CF555 anti-goat secondary antibody for 1 h at room temperature. Following wash and mount, images were acquired using a Zeiss LSM 710 laser-scanning confocal microscope.

Antibodies

Goat polyclonal anti-NLRP3 (ab4207, Abcam, Cambridge, MA), CF555 Donkey anti-goat IgG (20039-1, Biotium Inc, Fremont, CA).

Statistical analysis

Statistical analysis and graphs were performed using GraphPad Prism software (GraphPad V 10.0, Sacramento, CA). Data were presented as mean ± standard deviation (SD) and box and whiskers plot (min to max). Renal hemodynamic variables were adjusted for kidney weight, and renal vascular resistance (RVR) was calculated as the MAP to RBF/kidney wt ratio. Analysis of variance (ANOVA) followed by Holm-Sidak's *post hoc* test was used. Statistical significance was set at P value <0.05.

RESULTS

Early fluid plus NE resuscitation maintained systemic and renal hemodynamics in septic newborn pigs

Sepsis in newborn pigs developed as a hypodynamic disease state with dysregulated renal macro- and microcirculation. Compared to baseline values, MAP and COP decreased by more than 60% (Fig. 1) in the CLP nonresuscitated group. The resuscitation protocol prevented a MAP drop beyond 15 mmHg from baseline (Fig. 1A). It attenuated the decline in COP (Fig. 1B). In contrast to sham, renal vascular resistance proportionally increased in nonresuscitated sepsis, whereas RBF, coPf, mePf, and $mtPO_2$ progressively declined (Fig. 2). Resuscitation palliated sepsis-induced reduction in regional kidney perfusion but failed to mitigate the decrease in medullary oxygenation (Fig. 2).

Early fluid plus NE resuscitation mitigated sepsis-induced AKI in newborn pigs

To examine the hypothesis that sepsis impairs kidney function in newborn pigs, we measured GFR, plasma creatinine, and BUN levels in the pigs. Transdermal measurement of FITC-sinistrin kinetics showed delayed clearance in CLP nonresuscitated piglets compared to sham animals. The increased FITC-sinistrin half-life translated into a reduction in GFR of more than 70% in the CLP nonresuscitated group, whereas the sepsis-induced decline in GFR was attenuated in the CLP resuscitated piglets (Fig. 3A). Kidney injury was further confirmed in CLP nonresuscitated animals by the increase in plasma creatinine, BUN, and NGAL (an early marker of AKI), compared to sham (Fig. 3, B–D). The levels of these AKI biomarkers were decreased in CLP resuscitated piglets compared to the CLP nonresuscitated group (Fig. 3, B–D). Sepsis also resulted in isolated foci of renal tubular necrosis. Resuscitation significantly mitigated the sepsis-induced renal morphological injury (Fig. 4).

Early fluid plus NE resuscitation reduced sepsis-induced increases in plasma C-reactive protein and renal NLRP3 inflammasome in newborn pigs

Cecal ligation and puncture raised plasma CRP levels in the newborn pigs, which were significantly counteracted by fluid plus NE resuscitation (Fig. 5A). Fluorescence immunostaining revealed increased NLRP3 inflammasome levels in the kidneys of CLP nonresuscitated pigs, compared to both sham and CLP resuscitated animals (Fig. 5, B and C).

Early fluid plus NE resuscitation attenuated the levels of proinflammatory cytokines, tissue, and liver injury in septic newborn pigs

Proinflammatory cytokines [interleukin (IL)-1ra, IL-6, IL-10, IL-12, and IL-18] and tissue (LDH) and liver (ALT and AST) injury biomarkers were significantly elevated in the septic newborn pigs compared to sham, effects attenuated by resuscitation (Fig. 6).

DISCUSSION

This study evaluated the impact of early fluid plus NE resuscitation on systemic and renal hemodynamics in newborn pigs with septic-AKI secondary to CLP. Our findings suggest that 1) the newborn pig develops hypodynamic sepsis secondary to CLP, characterized by a progressive decline in MAP, COP, RBF, coPf, mePf, and $mtPO_2$; 2) CLP induces AKI characterized by a reduction in GFR, elevated biomarkers, and changes in kidney morphology; 3) fluid bolus plus NE resuscitation ameliorated the hypodynamic shock caused by sepsis, diminishes proinflammatory cytokines production and liver injury; and 4) although resuscitation restored renal perfusion, it did not affect the reduction in renal medullary tissue oxygenation.

Clinical and experimental data have demonstrated that the hemodynamic response of newborns to sepsis differs from that of adults. Septic adults develop a biphasic hemodynamic reaction characterized by an early hyperdynamic stage followed by a late hypodynamic stage (17,32). In contrast, neonates are more likely to develop a hypodynamic response from the onset of septic disease (17). Here, CLP in 3–7-day-old piglets induced sepsis with hypodynamic circulation throughout the 12 h of monitoring. Cardiac output, MAP, and RBF decreased, while renal vascular resistance increased. Because of the immaturity of the cardiac and autoregulatory mechanisms, newborns are inherently predisposed to reduced COP and higher vascular resistance (13,14,17). The hemodynamic findings from experimental studies are heterogeneous and highlight that age and species of animal models are determining factors in the translational approach of septic AKI. Various large animal models have closely mimicked the early human adult sepsis phenotype. *Escherichia coli* (*E. coli*) infusion to conscious sheep caused sepsis associated with hyperdynamic circulation, with preserved or increased RBF and decreased vascular resistance (33). Chvojka et al. (34) and Brandt et al. (35) observed increased COP and decreased systemic vascular resistance in fecal peritonitis-induced sepsis in the swine model. In pediatric animal models, like our data, newborn piglets subjected to lipopolysaccharide infusion developed hypotension and decreased cardiac index (36,37). In the comparative study by Seely et al. (38), the pediatric rat model of CLP-induced septic AKI showed significantly greater and faster hemodynamic deterioration than adult rats subjected to the same procedure. Although with a more moderate course, MAP and RBF also decreased in the adult rats, suggesting that adult rodents may be prone to hypodynamic sepsis when subjected to CLP (38,39).

In neonatal sepsis, CRP has been used as a diagnostic biomarker, although its increase is often not seen until 12 to 24 h after the start of infection (40,41). Our previous work demonstrated that plasma CRP levels were elevated significantly as early as 6 h after CLP in neonatal pigs, confirming the presence of sepsis (29). In this study, at 6 h, CLP-

induced decline in MAP, COP, and total RBF were significant, as opposed to changes in cortical and medullary perfusion and medullary oxygen tension. Our data show that macrocirculatory changes at 6 h have not yet substantially impacted renal microcirculation in this experimental model. However, 12 h after CLP, significant cortical and medullary hypoperfusion and hypoxia occurred. These events were concurrent with an impairment of kidney function in the CLP group, characterized by decreased GFR and increased plasma creatinine, BUN, and NGAL levels.

The reduction of cortical and medullary perfusion following the decline in systemic hemodynamics and RBF observed in the septic newborn pigs has been described in previous animal models of hypodynamic sepsis (38,39,42). With lower renal blood flow than adults and immature peripheral vasoregulation, neonates are more predisposed to kidney injury associated with changes in renal perfusion (13,14). The decrease in GFR in this study may arise from vasoconstriction caused by renin-angiotensin-aldosterone and sympathetic nervous system activation in response to sepsis-associated hypoperfusion and hypovolemia (43–46). Constriction of the arterioles and the vasa recta may further enhance renal microcirculatory hypoperfusion and hypoxia (44), which may initiate a vicious cycle of oxidative stress and inflammation that ultimately culminates in AKI (10,20,22,43). Because AKI also develops in hyperdynamic models of sepsis with increased RBF (34,47), there may be underlying mechanisms other than renal hypoperfusion that contribute to the development of neonatal septic AKI (20,42). The pathophysiology of newborn septic AKI likely revolves around a complex interplay between hemodynamic and immunologic factors. Increased renal NLRP3 inflammasome expression evidenced an inflammatory process in the kidneys of septic newborn pigs. This finding is corroborated by human kidney biopsies studies (48) and rat septic models (49), where upregulation of NLRP3 inflammasome expression corresponds to renal inflammation and multiorgan injury. Suppressing NLPR3 inflammasome expression improved renal function, reduced renal inflammation, and prevented multiorgan damage (48–50). Various sepsis-related factors associated with NLRP3 inflammasome in septic newborn pigs may include pathogen-associated and damage-associated molecular patterns (10,51).

The hallmark of septic AKI histopathology was formerly considered acute tubular necrosis (ATN) (52). However, the systematic review by Kosaka et al. (53) challenged this conjecture by revealing that in experimental septic AKI, ATN was uncommon and typically seen in low COP models. Histopathology in hyperdynamic models is characterized by nonspecific changes rather than ATN (34), consistent with findings in adult human postmortem tissue, where almost normal appearance or minor renal morphological changes were reported (54–56). Renal hypoperfusion associated with hypodynamic septic AKI has been described as the potential cause of ATN (53). While CLP in our model caused decreased COP and RBF, kidney morphological changes were minor. Of note, histopathological data on experimental newborn sepsis is scarce, and the findings described in human studies are from kidneys of adult deceased septic patients (55,56). It is uncertain whether the observed minor changes correspond entirely with histopathological features of human newborn septic AKI or are due to the 12-h duration of sepsis.

Resuscitation of the septic group followed current guidelines for managing sepsis in pediatric patients (23). Given that resuscitation with crystalloid fluids containing high chloride concentrations has been associated with a higher incidence of AKI (23,57), a balanced lactate Ringer was chosen over 0.9% saline. Fluid resuscitation followed by NE infusion successfully maintained MAP and COP within the established threshold. Norepinephrine increases MAP *via* α-adrenergic receptors, whereas the rise in COP occurs because of increased stroke volume *via* NE's modest β-adrenergic agonist effect (25). Fluid bolus plus NE resuscitation also attenuated the systemic proinflammatory cytokine production triggered by CLP, which may underlie the protection against AKI. Stolk et al. (58) observed that in both mice and humans, NE diminished proinflammatory cytokine release and enhanced anti-inflammatory cytokine production in response to lipopolysaccharide challenge. Immune cells possess β-adrenergic receptors, and the underlying mechanisms of anti-inflammatory activity of NE may include β-adrenergic stimulation and suppression of immunometabolism (58,59).

Furthermore, our data suggest liver injury in the septic newborn pigs, which was alleviated by fluid and NE resuscitation. Although intraportal NE infusion altered hepatocellular function in septic adult rats (60), vasopressor intervention in septic pigs protected against liver injury (61,62). A combination of species, age, or dosage may underlie the variability of liver responses to fluid and vasopressor intervention in sepsis. Additional studies are necessary to provide more insights into the mitigation of neonatal acute liver failure by early fluid and NE resuscitation.

Clinical and experimental data have shown that the timing of vasopressors may determine the response to resuscitation during sepsis treatment (24,63). The prospective study by Ospina-Tascon et al. (63) in adult patients with septic shock revealed that an early start of NE might benefit clinical outcomes by reducing the risk of fluid overload and shortening the time of sepsis-induced hypoperfusion. However, this vasopressor can also harm those patients who develop catecholamine-resistant septic shock if NE resuscitation is not discontinued (26). In cases of refractory hypotension, combined therapy using NE as the primary vasopressor alongside adjunctive terlipressin has been shown to effectively reduce the dosage of NE leading to improved clinical outcomes (64). Regarding sepsis-induced AKI, there is no consensus on the effect of NE on kidney function and microcirculation. Hyperdynamic models of sepsis have shown that in response to vasoactive drugs in sepsis, the microcirculation is dissociated from macrocirculatory changes, often resulting in exacerbated medullary hypoperfusion and hypoxia (33,34). This may again be a time-dependent phenomenon, as Giantomasso et al. (65) reported a favorable renal microcirculatory response to NE infusion in a model of early hyperdynamic sepsis in adult sheep. Norepinephrine improved systemic hemodynamics and urine output in preterm infants with sepsis (66). Here, we demonstrate that restoration of MAP and RBF in the early stages of sepsis was followed by recovery of cortical and medullary perfusion and a subsequent increase in GFR.

Noteworthy is that resuscitation failed to palliate the renal medullary hypoxia induced by CLP in the piglets in this study. The decrease in renal tissue oxygenation may result from an imbalance between local oxygen delivery and consumption (20). Oxygen delivery

is determined by the distribution of the renal blood flow to the cortical and medullary circulations (20). The renal medulla is more prone to hypoxia, congestion, and injury when renal blood flow is impaired during sepsis (20,42,43). The medullary vascular topography and the tubules' high oxygen demand render medullary tissue oxygen tension lower than the cortex's, even under healthy conditions (42,43). Moreover, diffusive and convective arteriovenous oxygen shunting may occur and enhance localized tissue hypoxia, with a greater impact on the medulla (20,22). On the other hand, oxygen consumption in the renal tissue is dominated by the energy required to drive tubular sodium reabsorption (20). Therefore, an increase in GFR and, thus, of sodium filtration during resuscitation may have increased oxygen consumption, promoting medullary hypoxia despite the recovery of renal medullary blood flow. Our findings contradict the study demonstrating that NE intervention in *E. coli*-induced sepsis in sheep restored blood pressure and preserved cortical perfusion and oxygenation but further aggravated medullary hypoperfusion and hypoxia (33). The apparent disparities in the results obtained from the study conducted by Lankadeva et al. (33) and our present study may arise from variations in species, age, or the sepsis model utilized.

The feasibility of measuring renal hemodynamics and obtaining kidney biopsies in critically ill infants is extremely limited. Therefore, the study of sepsis-induced AKI in newborns is challenging, and most of the understanding of this disease is primarily based on preclinical data (67). Because of its size and anatomical and physiological characteristics, the pig is an ideal model for studying neonatal disease, which has gained further interest given the advancement in xenotransplantation (68,69). Modeling newborn sepsis in the piglet provided the ability to evaluate hemodynamic parameters continuously in real-time throughout the 12 h of the study.

The CLP model induces polymicrobial sepsis in the experimental setting, which closely mimics the clinical features of human sepsis and, to some extent, resembles necrotizing enterocolitis occurring in neonates (67,69). As opposed to single-pathogen sepsis models, the polymicrobial challenge is considered the gold standard for neonatal sepsis because it mirrors the timeline progression of human sepsis more similarly (69). Following the "Minimum Quality Threshold in Pre-Clinical Sepsis Studies (MQTiPSS)" consensus (70), there are a few limitations in this study. This study focuses on the early phase of neonatal sepsis, encompassing only the first 12 h of an illness with a progression from days to weeks. Even though antimicrobial therapy comprises first-line treatment of septic neonates (23), it was excluded from this protocol so that changes generated solely by fluid bolus and NE resuscitation could be examined. Piglets in the study were subjected to general anesthesia, which may alter hemodynamics and promote reduced renal perfusion and oxygenation (42). Nevertheless, sedation and mechanical ventilation are standard-of-care treatments for critically ill septic neonates.

CONCLUSIONS

In conclusion, early sepsis in newborn pigs is associated with hypodynamic circulation and reduced renal blood flow. Early implementation of resuscitation to maintain blood pressure, COP, and kidney perfusion lessens the degree of inflammation, AKI, and liver

injury in the pigs. The discrepancy between medullary perfusion and oxygenation indicates that optimizing perfusion and blood flow may not correlate with improved medullary oxygen delivery in newborns. Further studies are necessary to investigate the outcome of resuscitation in the later stages of neonatal sepsis and the performance of additional management strategies addressing medullary hypoxia.

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Availability of data and materials:

The data and methods from this study are available from the corresponding author upon a reasonable request.

ABBREVIATIONS

AKI	acute kidney injury
ALT	alanine transaminase
ANOVA	analysis of variance
AST	aspartate aminotransferase
ATN	acute tubular necrosis
BUN	blood urea nitrogen
CLP	cecal ligation and puncture
COP	cardiac output
coPf	cortical perfusion
CRP	C-reactive protein
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein-isothiocyanate
GFR	glomerular filtration rate
IV	intravenous
LDH	lactate dehydrogenase
MAP	mean arterial pressure

mePf	medullary perfusion
mtPO$_2$	medullary tissue oxygen tension
NE	norepinephrine
NGAL	neutrophil gelatinase-associated lipocalin
RBF	total renal blood flow
RST	early fluid plus norepinephrine resuscitation
RVR	renal vascular resistance
SEM	standard error of the mean

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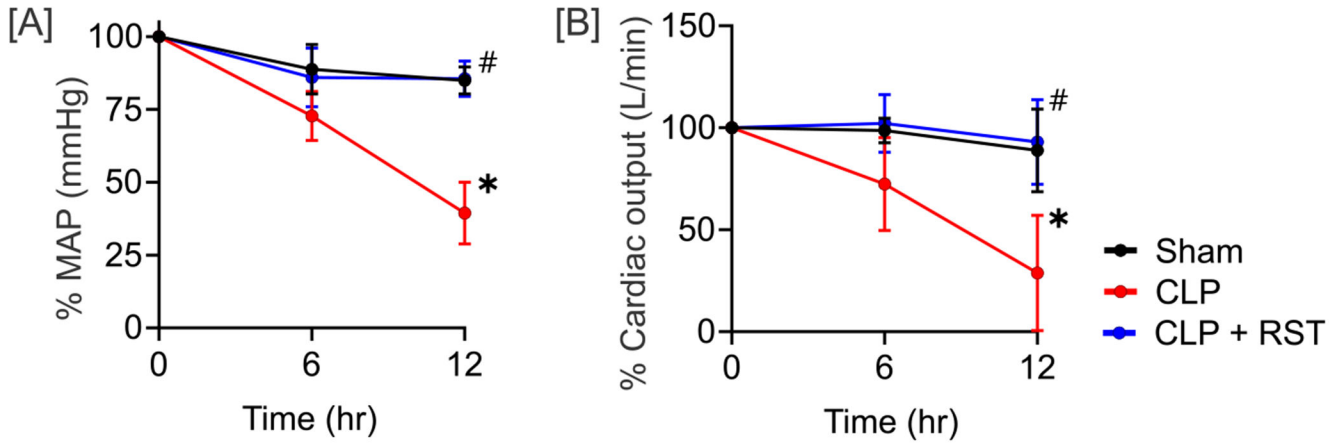

Fig. 1. Early fluid plus NE resuscitation maintained systemic hemodynamics in septic newborn pigs.

(A) MAP and (B) COP in newborn pigs subjected to sham operation, CLP, and CLP plus fluid and NE RST. $^{*}P < 0.05$ CLP *vs.* sham; $^{\#}P < 0.05$ CLP + RST vs. CLP. (MAP and COP: 6 and 12 h). Two-way ANOVA and Holm-šídák's multiple comparisons tests. ANOVA, analysis of variance; CLP, cecal ligation and puncture; COP, cardiac output; MAP, mean arterial pressure; NE, norepinephrine; RST, resuscitation.

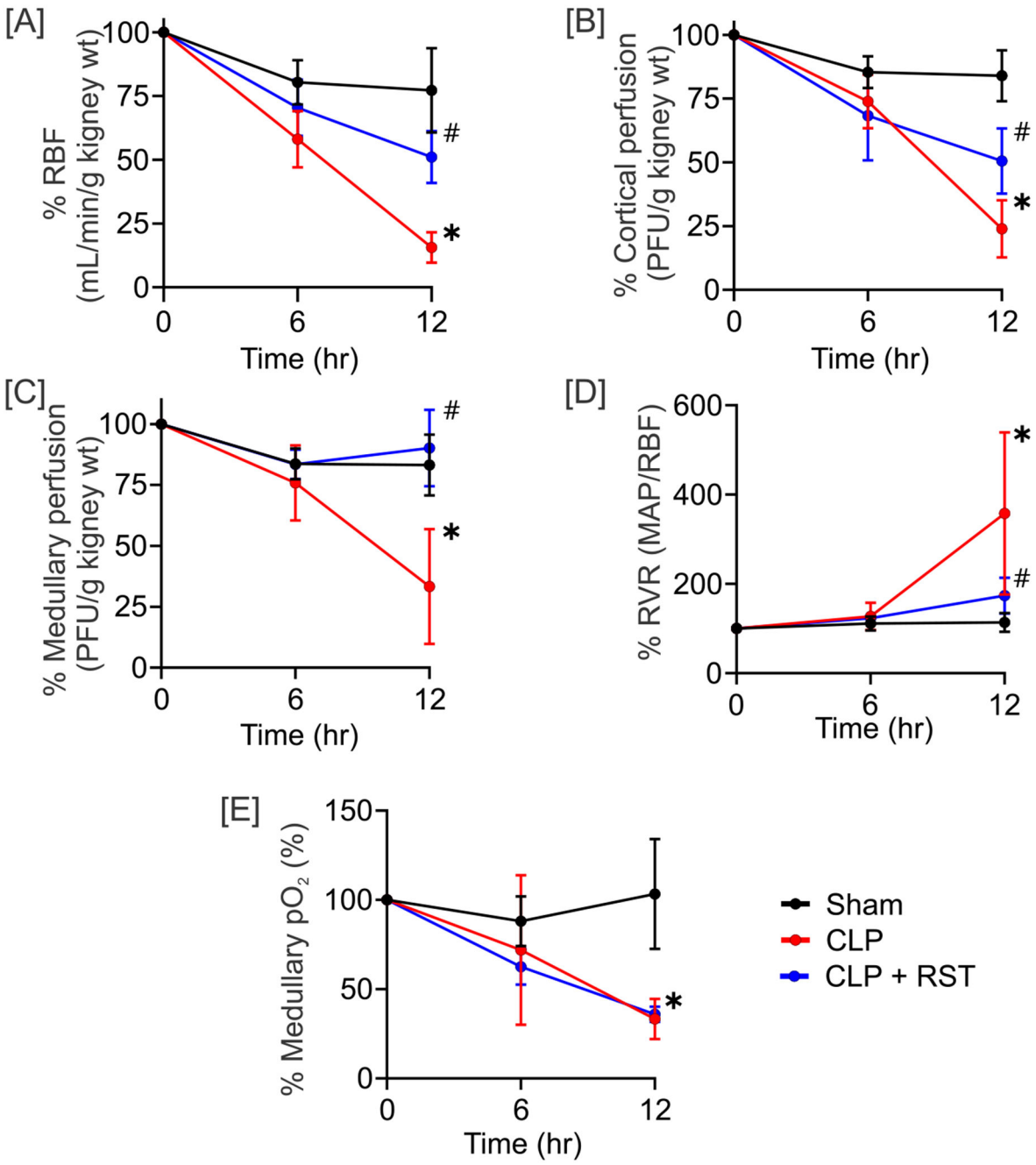

Fig. 2. Early fluid plus NE resuscitation maintained renal perfusion in septic newborn pigs.

(A) total RBF, (B) cortical perfusion, (C) medullary perfusion, (D) RVR, and E, medullary pO_2 in newborn pigs subjected to sham operation, CLP, and CLP plus fluid and NE RST. *P < 0.05 CLP *vs.* sham; #P < 0.05 CLP + RST *vs.* CLP (RBF: 6 and 12 h; cortical perfusion: 12 h; medullary perfusion: 12 h; RVR: 12 h); *P < 0.05 CLP *vs.* sham (medullary pO_2: 12 h). Two-way ANOVA and Holm-šídák's multiple comparisons tests. ANOVA, analysis of variance; CLP, cecal ligation and puncture; NE, norepinephrine; RBF, renal blood flow; RVR, renal vascular resistance; insert: NE, norepinephrine; RST, resuscitation.

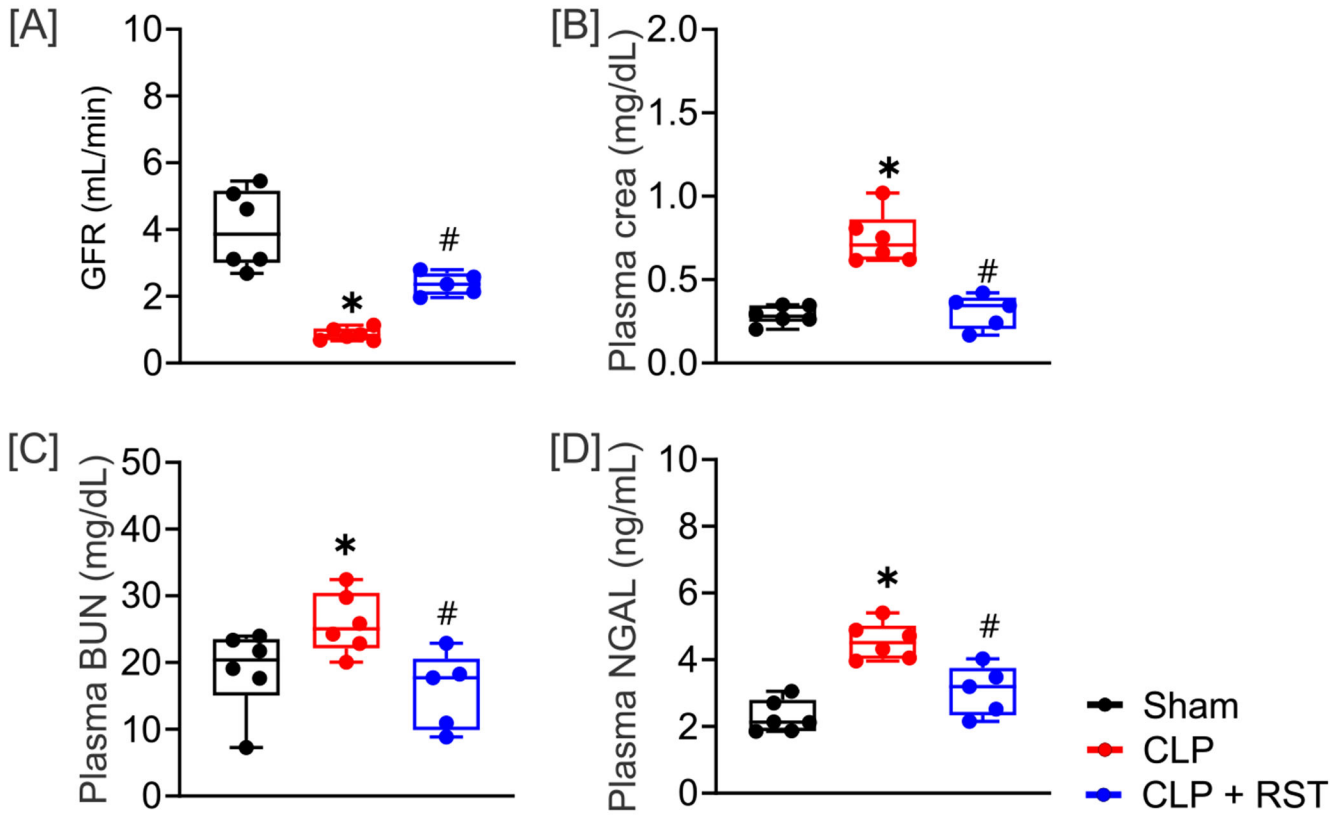

Fig. 3. Early fluid plus NE resuscitation mitigated sepsis-induced AKI in newborn pigs.

(A) GFR, (B) plasma creatinine (crea), (C) plasma BUN, and (D) plasma NGAL in newborn pigs subjected to sham operation, CLP, and CLP plus fluid and NE RST. *$P < 0.05$ CLP vs. sham; #$P < 0.05$ CLP + RST *vs.* CLP. One-way ANOVA and Holm-šídák's multiple comparisons tests. AKI, acute kidney injury; ANOVA, analysis of variance; BUN, blood urea nitrogen; CLP, cecal ligation and puncture; GFR, glomerular filtration rate; NGAL, neutrophil gelatinase-associated lipocalin; NE, norepinephrine; RST, resuscitation.

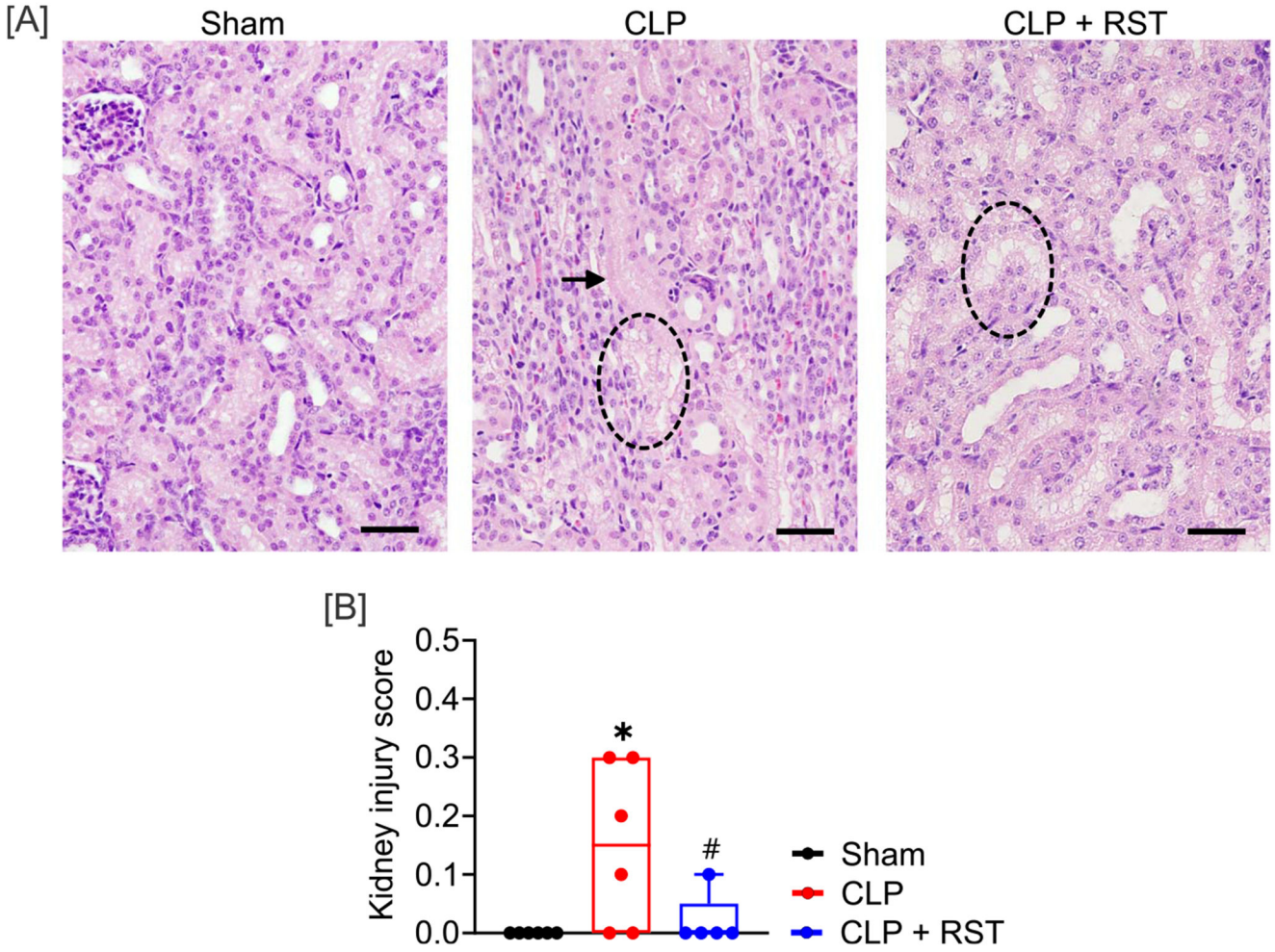

Fig. 4. Early fluid plus NE resuscitation attenuated sepsis-induced morphological changes in newborn pig kidneys.

(A) Hematoxylin and eosin staining showing isolated foci of tubular necrosis (arrow) and limited vacuolization (circled with dashed lines) at 20X objective magnification and (B) a summary of injury score in newborn pigs subjected to sham operation, CLP, and CLP plus fluid and NE RST. $^{*}P < 0.05$ CLP *vs.* sham; $^{\#}P < 0.05$ CLP + RST *vs.* CLP. One-way ANOVA and Holm-šídák's multiple comparisons tests. Bar = 50 μm. ANOVA, analysis of variance; CLP, cecal ligation and puncture; NE, norepinephrine; RST, resuscitation.

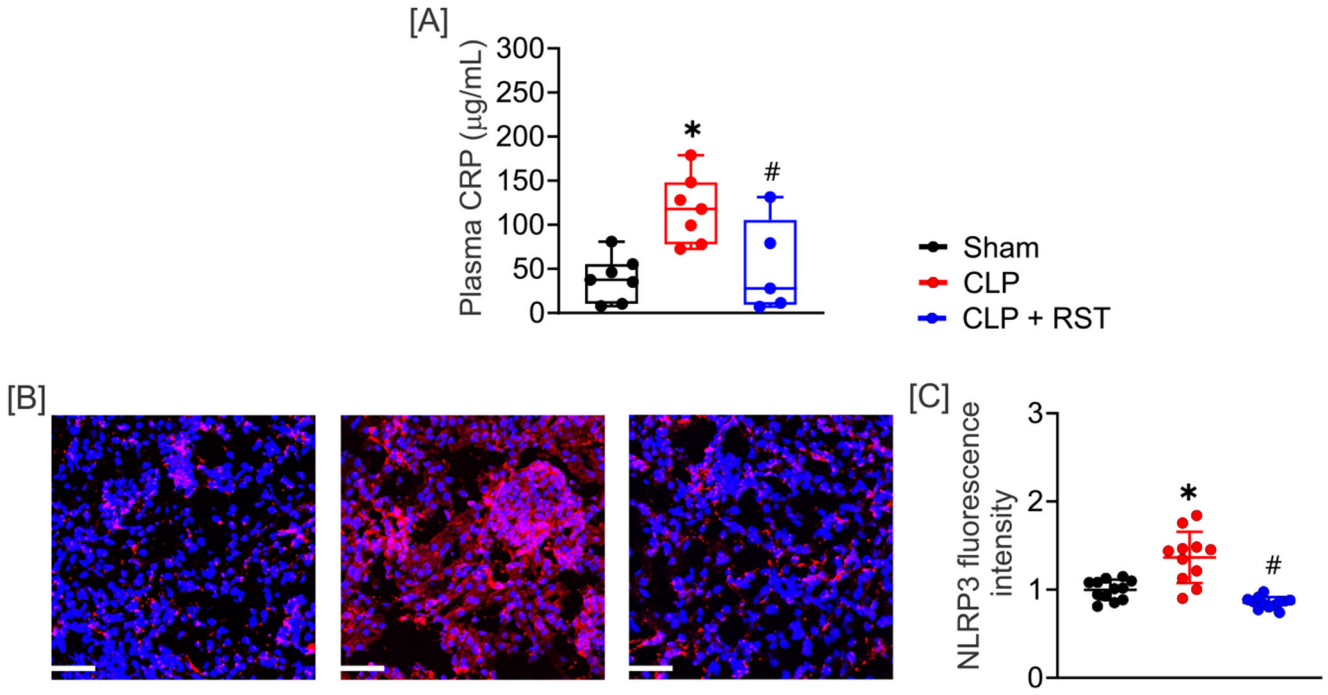

Fig. 5. Early fluid plus NE resuscitation reduced sepsis-induced increases in plasma CRP and renal NLRP3 inflammasome in newborn pigs.

(A) Plasma CRP and (B) representative renal NLRP3 immunostaining images, and (C) renal NLRP3 fluorescence intensity in newborn pigs subjected to sham operation, CLP, and CLP plus fluid and NE RST. $^{*}P < 0.05$ CLP *vs.* sham; $^{\#}P < 0.05$ CLP + RST *vs.* CLP. One-way ANOVA and Holm-šídák's multiple comparisons tests. Scale bar = 50 µm. ANOVA, analysis of variance; CLP, cecal ligation and puncture: CRP, C-reactive protein; NE, norepinephrine; RST, resuscitation.

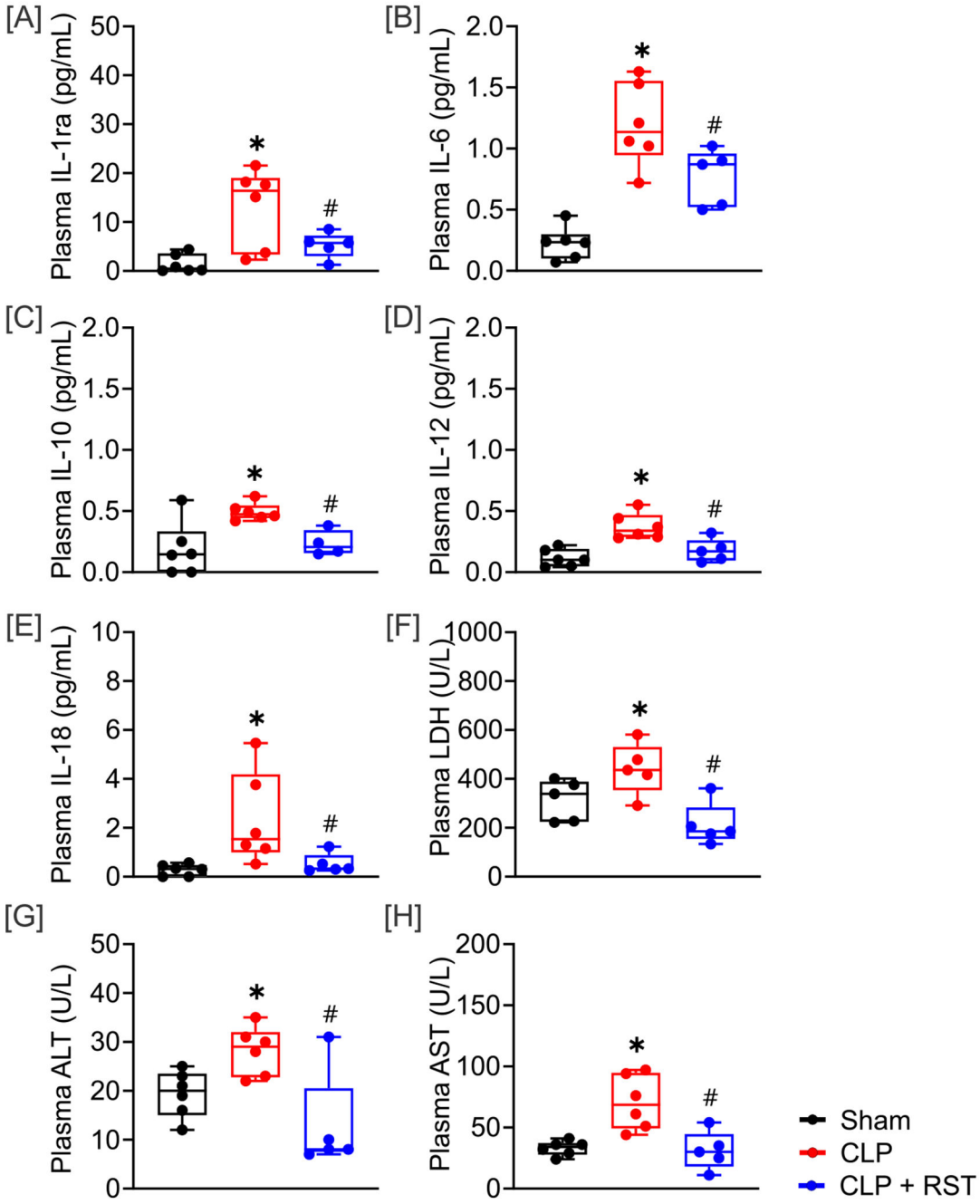

Fig. 6. Early fluid plus NE resuscitation attenuated the levels of proinflammatory cytokines, tissue, and liver injury in septic newborn pigs.

Plasma levels of A-E, IL-1ra, IL-6, IL-10, IL-12, and IL-18, F, LDH,G, ALT, and H, AST in newborn pigs subjected to sham operation, CLP, and CLP plus fluid and NE RST. $^{*}P < 0.05$ CLP *vs.* sham; $^{\#}P < 0.05$ CLP + RST vs. CLP. One-way ANOVA and Holm-šídák's multiple comparisons tests. ALT, alanine transaminase; AST, aspartate aminotransferase; ANOVA, analysis of variance; CLP, cecal ligation and puncture; IL, interleukin; LDH, lactate dehydrogenase; NE, norepinephrine; RST, resuscitation.