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EARLY MURINE POLYMICROBIAL SEPSIS PREDOMINANTLY CAUSES RENAL INJURY

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Abstract

Multiple organ failure in sepsis substantially increases mortality. This study examined if there was greater hepatic, pancreatic, splenic, or renal injury in mice that would die during sepsis induced by cecal ligation and puncture (CLP) compared with that of those that would survive. Mice were stratified into groups predicted to die (Die-P) or predicted to live (Live-P) in the first 5 days after CLP based on plasma interleukin 6 levels. Groups were sacrificed to harvest organs for histology. Separate animals were followed for survival with daily blood sampling to examine renal function. No significant histological evidence of organ injury was observed in either the Live-P or Die-P mice. Minimal hepatic injury occurred as plasma aspartate transaminase demonstrated less than a 2-fold increase over normal in both groups. In addition, pancreatic injury was minimal as there was also less than a 2-fold increase in plasma amylase levels. In contrast, blood urea nitrogen levels were nearly five times higher within 24 h in Die-P mice compared with those of mice predicted to live. Mice with blood urea nitrogen levels higher than 44 mg/dL had a 17.6 higher relative risk of dying (95% confidence interval, 4.5–69.4). Cystatin C, a more specific kidney function biomarker, was also elevated at 24 h after CLP. When the cystatin C levels were analyzed relative to the hours before death, rather than hours after CLP, they were also significantly increased in mice Dead by day 5 compared with those Alive after day 5. We conclude that limited liver, pancreas, and spleen injury develops during murine CLP-induced sepsis while significant kidney injury is present. The renal injury becomes worse closer to death.

Keywords

Renal injury; cystatin; BUN; AST; amylase; IL-6; biomarker

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INTRODUCTION

Mortality in sepsis remains intolerably high, with more than 200,000 patients dying each year in the United States alone (1). Severe sepsis has associated organ dysfunction (2). It is not completely understood how organ dysfunction and subsequent failure develop, and some hypothesize that they represent a protective mechanism because a decrease in function helps ensure the survival of organs faced with severe illness (3). However, the development of organ dysfunction is strongly correlated with increased mortality, and the more organs that fail, the higher the mortality, exceeding 80% for five failed organs (4).

The lungs are the organs that fail most frequently in septic patients (5), although a recent study published by our group has shown that mice die in the acute phase of cecal ligation and puncture (CLP)-induced sepsis without significant lung injury (6). After the lungs, the organ that fails most frequently in septic patients is the kidney (5), with as many as a third of the patients hospitalized with community-acquired pneumonia developing acute kidney injury (AKI) (7). In addition, approximately 50% of the patients who develop AKI have sepsis, making sepsis the leading cause of AKI in critical illness (8). The presence of both AKI and sepsis has an additive negative effect, with mortality rates about 70% (9), dramatically higher than those for patients who are only septic alone (1) or patients with AKI because of a different etiology (8). Acute kidney injury is not a uniform feature of the CLP model of sepsis as some investigators detect it with changes in serum blood urea nitrogen (BUN) and creatinine (10), whereas others do not (11). Acute kidney injury has also been reported to only be present in CLP sepsis in older animals (12). Recent publications elegantly described the dysfunction present in the renal tubules after CLP-induced sepsis (13) and the role of neutrophils in the development of AKI (14). More investigators document the development of AKI after CLP.

The hepatic system is not as frequently affected as the kidneys, but septic patients who have liver involvement have a very high mortality (1). Liver injury has been reported in the CLP model of sepsis (15). The pancreas has also been shown to be vulnerable during sepsis both in humans (16) and in animal models of the disease (17).

Given the controversy concerning the role of organ injury in sepsis mortality, as well as the issues concerning the utility of animal models (18), this study examined the association between sepsis mortality in the murine CLP model and injury of liver, pancreas, spleen, and kidney. The capacity to predict survival outcome in the first 5 days of murine intra-abdominal sepsis based on plasma interleukin 6 (IL-6) levels was used (19). Histology and markers of organ injury and function in mice predicted to die (Die-P) were compared with those of mice predicted to live (Live-P), with values from naive mice used as controls to determine normal ranges. Kidney function was also measured in a separate group of mice followed for survival until day 5 after CLP to allow comparison of results from mice within 24 h of their death with those from survivors. Overall, the intent of the study was not to demonstrate that CLP-induced sepsis causes organ injury but rather to determine what organs in mice predicted to die will have worse injury and could explain their survival outcome.

MATERIALS AND METHODS

Animals

Female adult ICR (Institute of Cancer Research) outbred mice (8 weeks old, 20–25 g body weight) were used in this study (Harlan–Sprague Dawley, Inc., Indianapolis, Ind). Females were chosen because they will not fight when housed together. Mice were housed in transparent plastic cages containing wood chip bedding (five animals in each cage). Each mouse was considered separately for data analysis. Mice were acclimated to the temperature (21°C–23°C)- and humidity (30%–70%)-controlled housing room programmed for a 12-h light-dark cycle for at least 72 h before the start of the experiment. These housing conditions were maintained throughout the experiment. The animals were provided standard rodent chow and water *ad libitum* for the duration of the study. The animals included in the study were cared for and used according to the provisions of the PHS Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals. The experiments were approved by Boston University Animal Care and Use Committee.

Sepsis model and sampling

Cecal ligation and puncture was performed according to our protocol (19), an adaptation of the original description (20). This model is based on the original description of the CLP model of sepsis (21, 22). Mice were initially anesthetized in an induction chamber by inhaling 5% isoflurane in 100% oxygen. After loss of consciousness, they were connected to a mask delivering 3% isoflurane in 100% oxygen for the duration of the surgical procedure. The cecum was ligated just below the ileocecal valve with 4-0 silk suture thread and double punctured with a 16-gauge needle to produce a mortality rate of approximately 50%. Immediately after surgery, mice were injected with 1 mL of normal saline subcutaneously (37°C) to replace fluid lost and help the recovery of body temperature. Pain medication was provided for the first 2 days after CLP (buprenorphine 0.05 mg/kg subcutaneously every 12 h; the first dose was given with the saline injection immediately after surgery and the other three doses were given with the antibiotic injections) and antibiotic treatment for the first 5 days after CLP (imipenem 25 mg/kg in 1 mL of D5W with Ringer's lactate solution, subcutaneously, every 12 h, starting at 2 h after surgery). The medication regimen was followed until the moment of scheduled sacrifice, death, or study completion in the surviving mice. Before the start of the experiments, animals were assigned to be sacrificed at 6 h (n = 20) or 24 h after CLP (n = 53) or followed for survival until day 5 after CLP (n = 99). A group of naive mice (no intervention) was also sacrificed and sampled in the same manner (n = 24). For histology, additional mice were sacrificed at 24 h (n = 13) and 48 h (n = 15). The final separation of these animals in analysis groups was performed as detailed in the following section of the Materials and Methods.

A 20-μL sample of blood was collected before administration of anesthesia in the mice sacrificed at 6 and 24 h after CLP, as well as the sacrificed control mice. The facial vein puncture technique was used, and blood was retrieved with an EDTA (ethylenediaminetetraacetic acid, 169 mM tripotassium salt)-rinsed pipette tip. The blood was immediately diluted 1:10 in phosphate buffer saline (PBS) with 1:50 EDTA, and plasma was collected after centrifugation (1,000g) at 4°C for 5 min. In 20 mice followed for

survival, blood was collected in a similar manner daily for the first 5 days after CLP (or until death), with the sample on day 1 collected at 6 h after the surgery. The plasma from these mice was used for measurements of BUN and cystatin C within 24 h of death. The other 79 mice followed for survival also had a 20- μ L blood sample collected at 24 h after CLP. These samples were used to measure BUN and perform receiver-operator characteristic analysis to predict survival to day 5 after CLP. This higher number of mice is usually used in this type of analysis. After anesthesia (87 μ g/g ketamine and 13 μ g/g xylazine in 250 μ L normal saline administered subcutaneously), blood was also collected from the retro-orbital venous plexus in mice that were sacrificed and was used to obtain undiluted plasma. This was used for the biochemical assays measuring aspartate transaminase (AST) and amylase that required a larger quantity of plasma. While still under anesthesia, euthanasia was performed by cervical dislocation, and death was confirmed by opening of the thoracic cavity. Liver, pancreas, spleen, and kidney tissue were collected from the sacrificed septic mice at 24 and 48 h after CLP, as well as from normal mice and fixed in 10% formalin.

Group stratification

To perform histological and some biochemical analyses, mice needed to be sacrificed, precluding determination of the actual survival outcome for these septic mice. Fortunately, information about their death or survival until after day 5 after CLP can be obtained by measuring plasma IL-6, a biomarker shown to be highly predictive of mortality in the CLP model of sepsis (19, 23). The septic mice sacrificed at 6 and 24 h were the same animals used for a recent publication that specifically addressed the issue of whether lung injury developed after CLP (6). Their survival outcome was predicted using a dual cutoff system for plasma IL-6 levels. At 6 h after CLP, plasma IL-6 levels higher than 14.8 ng/mL were used to predict death within the first 5 days (90% specificity), and levels less than 12.1 ng/mL predicted survival until after day 5 (95% sensitivity), with intermediate levels eliminated because of a higher chance of false-positive or false-negative results. Using this cutoff system, the 20 mice sacrificed at 6 h were separated into 11 predicted to die in the first 5 days after CLP (Die-P) and nine predicted to live until after day 5 after CLP (Live-P). A similar cutoff system was developed for plasma IL-6 values measured at 24 h after CLP, and the 53 mice sacrificed at that time point were separated in 14 Die-P (IL-6 higher than 12 ng/mL, 100% specificity) and 29 Live-P (IL-6 less than 1.5 ng/mL, 100% sensitivity) for biochemical analysis. The experiments were performed until up to 10 animals in each group were studied. Based on the IL-6 level at 24 h, mice included for histology were 8 Die-P and 5 Live-P at 24 h, as well as 7 Die-P and 8 Live-P at 48 h (for these experiments, five animals in each group were sought).

The animals in the survival experiments were separated into two groups: those that survived until after day 5 (Alive after day 5) and those that died within the first 5 days after CLP (Dead by day 5). This separation was based on the observed outcome and not on the plasma IL-6 prediction. To correlate BUN and IL-6 measurements, the values from individual mice were first log transformed and linear regression was performed.

Histopathology

Formalin-fixed kidney, pancreas, spleen, and liver tissues were embedded in paraffin and sectioned at 5 μ m for routine histology. Slides were stained with hematoxylin and eosin (H&E) as well as periodic acid-Schiff reagent for kidney histology. The renal histology was evaluated by Joel Henderson, MD, a board-certified pathologist with fellowship training in renal pathology. The remaining slides were scored by Daniel Remick, MD, a board-certified pathologist. Both pathologists were blinded for group affiliation when they performed their assessment of the slides.

Biochemical measurements

The concentrations of BUN, AST, and amylase were measured by standard clinical chemistry techniques using kits from Pointe Scientific, Inc. (Canton, Mich).

Cystatin C measurement

The concentration of plasma cystatin C was measured by enzyme-linked immunosorbent assay using commercially available matched antibodies and recombinant protein standards (R&D Systems, Minneapolis, Minn). The enzyme-linked immunosorbent assay was performed similarly to our previously described protocol (24), but blocking was performed with 150 μ L of Superblock Blocking Buffer in PBS (Pierce, Rockford, Ill), and the dilution buffer was PBS with 0.1% bovine serum albumin (Sigma Chemicals, St. Louis, Mo) and 0.005% Tween 20 (Surfact Amps, 10% Tween 20; Pierce, Rockford, Ill). The highest standard was 500 ng/mL, and six subsequent 1:3 dilutions were used to generate the standard curve.

Statistical analysis

Statistics were performed using Prism 5 (GraphPad Software). If more than two groups were compared, one-way analysis of variance followed by the Newman-Keuls posttest was used if the groups had normal distribution, whereas the Kruskal-Wallis test followed by Dunn posttest was used when the distribution was not normal. If two groups were compared, *t* tests were performed for normally distributed data and Mann-Whitney *U* tests were performed when data did not show normal distribution. A value of $P < 0.05$ was considered statistically significant. The receiver-operator characteristic curve analysis was performed to determine the capacity of BUN levels to predict the 5-day post-CLP survival outcome. The relative risk of death for the groups defined by the chosen cutoff (more than and less than 44 mg/dL) was also calculated using a contingency table analysis. To correlate BUN and IL-6 measurements, the values from individual mice were first log transformed and linear regression was performed.

RESULTS

Our model of CLP has an overall 28-day mortality rate of 50% (19, 25), and most of the deaths in the early phase (days 1–5) occur within the first 3 days. Therefore, samples collected at the 24-h time point from an animal predicted to die will likely be within 2 days of that outcome. There were no significant morphological changes observed in the liver, pancreas, or kidney in Live-P or Die-P mice compared with normal histology at 24 h (Fig. 1)

or 48 h (see Figure, Supplemental Digital Content 1, at <http://links.lww.com/SHK/A187>) after CLP. No significant histological differences were seen in liver, pancreas, or kidney specimens from Live-P or Die-P mice at 48 h after CLP compared with those of normal animals. Both Live-P and Die-P spleens demonstrated characteristic postsepsis changes, as detailed in Figure 1. Hematoxylin and eosin slides were evaluated by light microscopy and photographed at 200× magnification. At 48 h, n = 8 for Live-P and n = 7 for Die-P. For control mice, n = 8. The spleens from the same CLP mice demonstrated extensive lymphocyte apoptosis, starry sky appearance, and congestion compared with those of naive mice. Hotchkiss et al. (26) have previously described significant splenic changes after CLP sepsis.

Histology is a relatively crude method to evaluate organ or cell injury. Therefore, biochemical tests were performed to evaluate liver, pancreas, and kidney damage. Normal values were established using plasma samples from naive mice, the same animals used for histology. Liver injury was assessed by measuring AST levels. At 6 h after CLP, the levels of AST were at the higher end of the normal range (as defined in Table 1) in both Live-P and Die-P (Fig. 2). There was a statistically significant increase in plasma AST by 24 h in both groups, with slightly higher levels in Die-P mice. However, the levels were less than 2-fold higher than normal for both groups, indicating only mild liver injury.

Serum amylase levels are commonly used to detect pancreatic injury. Amylase levels were slightly above normal 6 h after CLP in both Live-P and Die-P mice (Fig. 3). However, at 24 h, there was a significant increase in amylase levels for the Die-P mice. Because the levels were only 60% higher than normal, this probably indicates a statistically significant but biologically irrelevant pancreatic injury, with much higher levels being typically detected in the presence of severe pancreatitis.

Kidney injury has been traditionally detected by measurements of BUN and creatinine (27). At 6 h after CLP, the average levels of BUN for Live-P mice were slightly above the upper limit of normal (Fig. 4A). In Die-P at the same time point, they were significantly higher than the Live-P values and were almost 2-fold above normal. By 24 h, the BUN levels returned to normal in Live-P mice, but they continued to rise in Die-P mice to a level almost five times higher than Live-P at the same time point. These changes in BUN are similar to the alterations observed in plasma levels of IL-6 (25, 28). We compared BUN and IL-6 and demonstrated a close correlation between the two measurements (Fig. 4B).

Interleukin 6 levels predict whether mice will die before the end of day 5 after CLP (23), but the exact day of death is still uncertain. To measure the BUN levels closer to the time of death, mice underwent CLP and then had blood samples collected daily until death or the end of the experiment on day 5 after CLP. The BUN levels recorded within the last 24 h of life (last sample collected before death) for the animals that succumbed to sepsis (Dead by day 5) were above normal and significantly higher than the levels recorded at corresponding time points in mice that survived past day 5 (Fig. 5A).

The considerable difference between Live-P and Die-P murine BUN levels at 24 h after CLP observed in Figure 4 suggests that BUN may be able to predict sepsis survival. To test this

idea, CLP was performed and animals were followed for survival until after day 5 and retrospectively separated into two groups: Dead by day 5 and Alive after day 5. Note that these groups were not the same as those predicted to live or die based on plasma IL-6. The BUN levels were measured in samples collected at 24 h after CLP from these mice (Fig. 5B). The mean BUN levels recorded were virtually identical to the BUN levels in mice separated based on IL-6 survival prediction in Figure 4 (Alive after day 5 versus Live-P and Dead by day 5 versus Die-P). A receiver-operator characteristic curve analysis was performed based on the data in Figure 5, and the area under the curve was 0.96, indicating excellent prediction capability (Fig. 5C). A cutoff level of 44 mg/dL predicted the outcome, with 92% sensitivity and 85% specificity. Mice with BUN levels higher than 44 mg/dL had a relative risk for mortality of 17.6 (95% confidence interval, 4.5–69.4) compared with the mice with lower levels. The data in Figures 4 and 5 indicate that kidney injury develops in the first 24 h of the evolution of sepsis in mice that will succumb to the disease.

A more specific marker of kidney function, cystatin C, was also measured at 24 h after CLP. Within 24 h, the cystatin C levels of both Live-P and Die-P mice were higher than those in normal mice (Fig. 6A). The difference between the Live-P and Die-P was small but statistically significant. Levels of this biomarker are considered to increase early in the development of kidney injury (27), and this small increase at 24 h could be exacerbated closer to death in Die-P mice if development of injury is a fast preterminal event.

To test if increased kidney injury develops closer to death in septic animals, mice were followed for survival until after day 5 after CLP and had their daily blood samples used to measure cystatin C (same mice as in Fig. 5A). The cystatin C levels were measured in samples collected within 24 h of death from mice that succumbed during the first 5 days after CLP and compared with levels measured at matched time points in mice that survived until after day 5. As seen in Figure 6B, the mean of the cystatin C levels of the survivors was similar to normal value. The nonsurvivors, however, had significantly higher levels than the survivors and normal mice. The mean for the levels in Dead by day 5 was 2.4 times higher than the mean of the normal mice. Taken together, the data presented in Figure 6 indicate that more significant kidney injury develops closer to death in septic mice, particularly in the last 24 h.

DISCUSSION

Liver, pancreas, spleen, and kidney injuries were evaluated in the murine CLP model of sepsis to determine if their presence could explain the pathophysiology of why some animals die while others survive. The histology of liver, pancreas, and kidney tissues was unremarkable 24 or 48 h into the evolution of sepsis for both mice predicted to die and survive. Other than changes such as lymphocyte apoptosis in the spleens of both groups, the lack of histological findings is consistent with the situation in human sepsis (26) and confirms CLP as a model for sepsis research that more closely reflects the human pathology. It should be noted that the current study compared the histology of septic mice predicted to die with that of septic mice predicted to live, whereas most previous studies compare septic mice with normal mice. The similarities between the CLP model of sepsis and human sepsis are shown in the Table, Supplemental Digital Content 2, at <http://links.lww.com/SHK/A188>.

Our group has also recently published that there is no significant lung injury or dysfunction in the murine CLP model of sepsis (6). In the current study, some biomarker measurements of organ injury were elevated, particularly at 24 h after CLP in the mice predicted to die. Biologically significant injury, however, was only found for the kidneys, especially within the last 24 h of life for the animals that died after CLP. Although the association of sepsis and AKI is clearly established (8), our findings highlight the importance and timing of kidney injury in the murine CLP sepsis model.

Increased levels of AST, amylase, BUN, and cystatin C indicate a level of multiple organ injury. Two alternate hypotheses exist to explain the findings. Multiple small insults to different organ systems could act in combination/synergy to produce lethality in murine sepsis. However, the levels measured would not be expected to indicate substantial alterations in organ function. More likely, intra-abdominal sepsis causes a mild degree of multiple organ damage but not overt failure of the liver, pancreas, or kidney at this time point. In models of acetaminophen-induced acute liver injury, AST levels of 15,000 U/L were reported at 24 h, representing more than a 50-fold increase over those seen in our septic mice (29). Amylase in lethal acute pancreatitis measured more than 12,000 U/L, higher than an 8-fold elevation compared with our CLP mice (30). In our current study, BUN levels of 90 mg/dL approached those seen in a lethal model of renal ischemia-reperfusion injury of about 98 mg/dL at 24 h (31). There may be progressive organ injury closer to death, and the measurements for cystatin C indicated that more significant kidney injury did develop in 24 h before death in nonsurviving mice. Obtaining histological evidence for this increased kidney injury is not feasible with current methods. The prediction based on plasma IL-6 levels indicates whether the animal will die in the first 5 days after CLP but not the exact moment.

The pancreatic blood supply seems to be affected earlier than that of other organs in sepsis (32), raising the possibility of increased injury. A small elevation in amylase levels was detected for Die-P mice at 24 h, indicative of only limited injury at this time point in the evolution of sepsis (33). The levels reached are consistent with previously described findings in sepsis studies using the murine CLP model (34). Similarly, the AST levels suggest only mild liver injury at 24 h and only slightly worse in mice predicted to die. A recent study does show an increasing trend in AST levels until 48 h after CLP in nonsurvivors, but this was in the setting of a double-hit model (sepsis after trauma/hemorrhage) (35). Even though closer to death, the level of kidney injury seems significant, because 24 h before death, there was a substantial increase compared with those of mice that survived, as indicated by cystatin C levels in this study. Examining these organs together, it is apparent that a small degree of multiple organ injury manifests at 24 h into the progression of CLP sepsis, particularly in the animals that were going to die. Because limited blood volume can be collected in our nonlethal serial sampling technique, daily monitoring of AST and amylase levels was not possible with our techniques. Consequently, we could not test if the progression to overt kidney injury in dying mice is also associated with severe liver and pancreas injury throughout the acute phase.

Historically, serum creatinine has been the most frequently used endogenous marker for estimating the glomerular filtration rate (GFR). However, it is not considered ideal because

the levels are influenced by factors, such as diet, muscle mass, sex, race, and age; it is actively secreted by the proximal tubules that can lead to a 10% to 40% overestimation of the GFR; and it does not work well for detecting smaller decreases, that is, less than 40% of GFR (36). Cystatin C, a 13-kd endogenous cysteine proteinase inhibitor, has recently been proposed to be a better marker for GFR, and two meta-analyses that looked at more than 100 studies comparing it with serum creatinine have confirmed this (37, 38). Cystatin C has previously been used to diagnose AKI in the CLP model (39). It is constantly produced by a housekeeping-type gene in all nucleated cells, without being affected by muscle mass, sex, or age. It is freely filtered by the glomeruli and not secreted by tubular cells, but it is reabsorbed and fully catabolized by proximal renal tubular cells. Serum cystatin C has been shown to be the best for early detection of AKI or for diagnosis of already established AKI (27). Cystatin C has also been shown to be a more sensitive and earlier marker for renal dysfunction than BUN and creatinine in mouse models of AKI (40).

Some of the formulas based on cystatin C indicate a linear relation between GFR and the reciprocal of serum cystatin C (41). This indicates that the increase in cystatin C levels seen in this study for septic mice within 24 h of death would correspond to a decrease of more than 50% in GFR, indicative of injury by the RIFLE classification criteria (42). Although this study did not attempt to uncover mechanisms of kidney injury in sepsis, a better understanding of the moment when it happens after CLP should help future mechanistic studies. A 2010 report of cystatin C levels in CLP sepsis found no difference between sham- and CLP-operated rats, but the values obtained were about 10 times lower than in our study (43). This could be an interspecies difference because our levels are more in line with previous reports of kidney injury in mice (39, 40) or caused by a difference in illness severity modulated by the lethality of the septic insult.

As a marker of kidney function, BUN has been considered to be even less specific than creatinine because its levels are affected, apart from glomerular filtration, by tubular reabsorption and also by the rate of its production, which in turn is influenced by protein intake, tissue protein catabolism, gastrointestinal bleeding, and corticosteroid therapy. As such, urea nitrogen is a reflection of many simultaneous pathological processes. The timing of some of these processes in CLP sepsis could explain why the BUN values measured in the last 24 h of life decrease when compared with those measured at 24 h after CLP, while the more specific marker of kidney function, cystatin C, increased in the same time span. Blood urea nitrogen levels higher than 40 mg/dL on admission to the intensive care unit were associated with increased mortality in critically ill patients, particularly at 30 days but even up to a year (44). The same BUN levels were also associated with a higher risk of positive blood cultures. From the data obtained in our study, a level of 44 mg/dL at 24 h after CLP was highly predictive of 5-day mortality, indicating that BUN could be useful as a biomarker for sepsis mortality. In addition, there was a close correlation of BUN and IL-6 (a well-established biomarker for sepsis mortality).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Organ histology. There were no significant morphological changes in liver, pancreas, or kidney in either Live-P or Die-P mice at 24 h after CLP as compared with normal mice. However, the spleens from Live-P and Die-P mice exhibited typical postsepsis characteristics such as lymphocyte apoptosis, “starry sky” appearance from megakaryocyte loss, and congestion. Hematoxylin and eosin–stained slides were evaluated by light microscopy, and photographs were taken at 200× magnification. At 24 h, n = 5 for Live-P and n = 8 for Die-P. For control mice, n = 8.

Fig. 2. Plasma AST levels. Septic mice, both Live-P and Die-P, had plasma AST levels near the upper limit of normal in both groups

The AST levels continued to increase 24 h after CLP, indicative of only mild liver injury. At 6 h, $n = 9$ for Live-P and 11 for Die-P; at 24 h, $n = 27$ for Live-P and $n = 14$ for Die-P.

Results are shown as mean $\pm$ SEM. The dotted horizontal lines indicate the normal interval (values given in Table 1). * $P < 0.05$ compared with both 6-h groups; # $P < 0.05$ compared with all other groups.

Fig. 3. Plasma amylase levels. The Die-P mice had higher serum amylase at 24 h, with levels reflecting only mild pancreatic injury
 At 6 h, n = 9 for Live-P and 11 for Die-P; at 24 h, n = 27 for Live-P and n = 14 for Die-P. Results are shown as mean $\pm$ SEM. The dotted horizontal lines indicate the normal interval (values shown in Table 1). [#] $P < 0.05$ compared with all other groups.

Fig. 4. Plasma BUN concentrations and correlation with IL-6. A, Live-P mice plasma BUN levels were slightly above normal at 6 h but returned to the normal range by 24 h

In contrast, in Die-P mice, the plasma BUN was already increased by 6 h and continued to increase at 24 h after CLP. At 6 h, $n = 9$ for Live-P and $n = 11$ for Die-P; at 24 h, $n = 27$ for Live-P and $n = 14$ for Die-P. Results are shown as mean $\pm$ SEM. The dotted horizontal lines indicate the normal interval (values provided in Table 1). * $P < 0.05$ compared with both 6-h groups; # $P < 0.05$ compared with all other groups; \$ $P < 0.05$ compared with Live-P at 6 h. B, Each symbol represents the BUN and IL-6 values for an individual mouse, and the straight line shows the linear regression. There is a close correlation between the two measurements. $P < 0.001$.

Fig. 5. Renal injury precedes death. For these studies, daily serial blood samples were collected, and the mice not sacrificed and groups were determined by the status at day 5 (alive or dead). A, Death is preceded by high BUN levels. Blood urea nitrogen level was measured in the last daily sample taken before death (within 24 h) for the mice in the Dead by day 5 group and in samples at corresponding time points in the Alive after day 5 group. Results are shown as mean $\pm$ SEM. $n = 12$ for Alive after day 5 and $n = 7$ for Dead by day 5. $*P < 0.05$ when compared with Alive after day 5. B, Blood urea nitrogen level at 24 h after CLP is predictive of 5-day mortality. Each symbol is the result from an individual mouse, and the horizontal line in each column is the mean of the group. The two dotted horizontal lines are the range for normal mice. A predictive discrimination level of 44 mg/dL (longest solid line) gives high sensitivity (92%) and specificity (85%). $n = 53$ for Alive after day 5 and $n = 26$ for Dead by day 5. $P < 0.001$ comparing the two groups. C, Receiver-operator characteristic curve for BUN at 24 h after CLP provides an area under the curve = 0.96.

Fig. 6. Plasma cystatin C concentrations indicate renal injury

A, Mice were stratified into Live-P and Die-P on the basis of IL-6 levels. Both Live-P and Die-P mice had slightly increased cystatin C levels 24 h after CLP compared with normal mice. $n = 15$ for each group. $*P < 0.05$ compared with normal; $\#P < 0.05$ compared with normal or Live-P. **B, Marked kidney injury develops within 24 h before death.** Mice had blood samples collected daily and were followed until day 5 after CLP. Cystatin C was measured in the last daily sample taken before death for the mice in the Dead by day 5 group and in samples at corresponding time points in the Alive after day 5 group. $n = 12$ for Alive after day 5 and 7 for Dead by day 5. $\#P < 0.05$ compared with normal or Alive after day 5.

Table 1

Normal interval determination

Test	n	Low	High	Mean	95% CI	Range (mean $\pm$ 2 SD)
AST, IU/L	24	43.1	177.7	77.5	63.2–91.8	9.8–145.2
Amylase, U/L	24	505.2	850.8	664.5	625.4–703.6	479.5–849.5
BUN, mg/dL	24	12.0	26.7	18.1	16.5–19.8	10.3–25.9
Cystatin C, ng/mL	15	85.5	151.5	127.0	116.3–137.7	88.2–165.8

The normal interval was defined as the mean $\pm$ 2 SD for the values obtained from each test in a group of naive mice (no intervention).

95% CI, the 95% confidence interval of the mean; high, the highest value measured in the group of normal mice; low, the lowest value measured in the group of normal mice; n, the number of normal mice used for each test.