Effects of Ketamine on Pulmonary Inflammatory Responses and Survival in Rats Exposed to Polymicrobial Sepsis

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ABSTRACT - PURPOSE. Ketamine is reported to suppress production of proinflammatory cytokines and activity of nuclear factor-kappa B (NF-κB) after lipopolysaccharide (LPS) stimulation. Our study was designed to investigate the effects of ketamine on pulmonary inflammatory responses and survival in a clinically relevant model of polymicrobial sepsis, induced by cecal ligation and puncture (CLP). **METHODS.** After the induction of sepsis or sham-operation, animals were treated with ketamine (0.5, 5 or 10 mg/kg) or saline (10 ml/kg) at 3h after operation. At 6 h post-operation, the levels of tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6, activity of NF-κB, expression of Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4) of the lungs were measured. And the mortality was recorded for 7 days. **RESULTS.** TNF-α and IL-6 production, NF-κB activity, TLR2 and TLR4 expression in rat lungs were increased after CLP. Ketamine at the doses of 5 mg/kg and 10 mg/kg suppressed CLP-induced elevation of TNF-α and IL-6 production, NF-κB activity and TLR2 expression. Ketamine 0.5, 5 and 10 mg/kg inhibited TLR4 expression in sepsis. Ketamine 5mg/kg and 10 mg/kg after CLP improved the survival of rats. **CONCLUSIONS.** Ketamine at sub-anesthetic doses could suppress the production of inflammatory cytokines such as TNF-α and IL-6, attenuate NF-κB activity, and inhibit TLR2 and TLR4 expression in polymicrobial sepsis. These anti-inflammatory effects of ketamine may correlate with improved survival in sepsis.

INTRODUCTION

Sepsis is a devastating medical condition that often results in the development of multiple organ failure (MOF), and is considered as a leading cause of death in critically ill patients. It has been reported that the lung is usually the first organ to fail during sepsis, and pulmonary failure is a common cause of mortality following sepsis (1-2). Excessive activation of the inflammatory cascade has been suggested to be a major factor that contributes to the injury of various organs in sepsis, including the lung (3).

Ketamine has been widely used in clinical anesthesia, especially for septic or severely ill surgical patients, because of its effect in maintaining cardiovascular function (4). Moreover, it has been reported that ketamine can suppress production of cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), after lipopolysaccharide (LPS) stimulation in vitro (5-7). Previous reports from our laboratory and others also revealed that, ketamine inhibited the elevation of inflammatory cytokines and nuclear factor-kappa B

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(NF-κB), which could regulate the expression of many genes of inflammatory cytokines (8), during LPS-induced sepsis in vivo (9-11). Cecal ligation and puncture (CLP) is a model of polymicrobial sepsis, which has been considered to reproduce inflammatory responses and pathological sequela of clinical sepsis closely, and has been used successfully in many experiments (12-15).

Our study was to investigate effects of ketamine on inflammatory cytokines, such as TNF-α and IL-6, and nuclear factor-kappa B (NF-κB), in the lungs of rats during CLP-induced sepsis. Further more, we tried to examine the effects of ketamine on Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4), which may mediate the activation of NF-κB and lead to production of cytokines (16), in a CLP model of polymicrobial sepsis. In addition, we intended to study the effect of ketamine on survival in the CLP model of sepsis.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (250-300 g body weight) were obtained from Shanghai Animal Center, Shanghai, China. The rats were exposed to 12 h of light and 12 h of darkness each day, and were given access to food and water ad libitum. The experimental procedures were approved by the Institutional Animal Ethics Committee.

Experimental protocol

Rats were randomly assigned to six groups (6 rats/group): sham-operation plus normal saline (NS, 10 ml/kg), CLP plus NS (10 ml/kg), CLP plus ketamine (0.5 mg/kg), CLP plus ketamine (5 mg/kg), CLP plus ketamine (10 mg/kg) and sham-operation plus ketamine (10 mg/kg).

Sodium pentobarbital (50 mg/kg, Sigma Chem Co., St. Louis, MO) was injected intraperitoneally (i.p.) to anesthetize the animals before the surgical procedures. Polymicrobial sepsis was induced by CLP as described previously (12-14, 17). In brief, a midline incision about 2 cm was made on the anterior abdomen. The cecum was carefully isolated and the distal 20% was ligated. Then the cecum was punctured twice with a sterile 21-gauge needle, and was squeezed to extrude the cecal contents from the wounds. The cecum was placed back and the abdominal incision was closed. The sham control animals were treated in an identical manner, but no cecal ligation or puncture was performed. All rats were administered 1ml sterile NS subcutaneously (s.c.) immediately after surgery for fluid resuscitation.

At 3h after operation, rats were treated with ketamine (ketamine hydrochloride, Hengrui, Inc., Nanjing, China) 0.5, 5 or 10 mg/kg (i.v.) or NS (10 ml/kg, i.v.). At 6 h post-operation, the rats were sacrificed. Then the lungs of rats were harvested and stored in liquid nitrogen for later use.

Enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α and IL-6 of rat lungs were measured by commercial ELISA kits (TNF-α, Diaclone Research, France; IL-6, Biosource Europe SA, Belgium) following the manufacturers' instructions. The values of TNF-α and IL-6 were expressed as pg/mg protein.

Electrophoretic mobility shift assay (EMSA)

Nuclear protein of rat lungs was extracted and quantified as previously described (18). EMSA was performed using a commercial kit (Gel Shift Assay System, Promega, Madison, WI) according to previous studies of our laboratory (6). Briefly,

dsDNA oligonucleotide probe for NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3') was synthesized commercially. The NF-κB oligonucleotide probe was end-labeled with [γ-^{32}P] ATP (Free Biotech, Beijing, China) with T4-polynucleotide kinase. Binding reactions were performed using nuclear extract protein (80 μg) preincubated in a binding buffer (9 μl), containing 10 mmol/l Tris-HCl (pH 7.5), 1 mmol/l $MgCl_2$, 50 mmol/l NaCl, 0.5 mmol/l EDTA, 0.5 mmol/l DTT, 40 ml/l glycerol, and 0.05 g/l of poly-(deoxyinosinic deoxycytidylic acid) for 15 min at room temperature. After addition of the 1 μl ^{32}P-labeled oligonucleotide probe, the incubation was continued for 30 min at room temperature. Reaction was stopped by adding 1 μl of gel loading buffer, and the mixture was subjected to non-denaturing 40 g/l polyacrylamide gel electrophoresis in 0.5×TBE buffer. The gel was dried and exposed to X-ray film (Fuji Photo Film Co., Ltd., Japan) at -70°C. NF-κB activity was measured by densito-metry, using Bandleader 3.0 software (Magnitec Ltd., Israel).

Reverse-transcription polymerase chain reaction (RT-PCR)

The expression of TLR2 and TLR4 mRNA in rat lungs was assessed by RT-PCR. Total RNA was extracted with TriPure Isolation Reagent (Roche Molecular Biochemicals, Basel, Switzerland), and the concentration was determined by spectrophotometric optical density measurement at 260 nm. 2 μg of RNA was used in reverse transcription (RT) with a Reverse Transcription System Kit (Promega, Madison, WI, USA) according to the protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a normalization control. The primer sequences were: TLR2(sense): 5' - A C A G C T A C C T G T G T G A C T C T C C G C C - 3', TLR2 (antisense): 5'-GGTCTTGGTGTTCAT TATCTTGCGC-3'; TLR4 (sense): 5' - T G G A T A C G T T T C C T T A T A A G - 3', TLR4 (antisense): 5'-GAAATGGAGGCACCCCTTC-3'; GAPDH (sense): 5' - T C C G C C C C T T C C G C T G A TG-3', GAPDH (antisense): 5' - C A C G G A A G G C C A T G C C A G T G A - 3'.

Polymerase chain reaction (PCR) was performed with 100 μl reaction mixture of 2 μl of RT product, 1.5 mmol/l $MgCl_2$, 2.5 U Taq DNA polymerase, 100 μmol/l dNTP, 0.1 μmol/l primer, and 1xTaq DNA polymerase magnesium-free buffer (Promega, Madison, WI, USA). Two drops of mineral oil (Sigma Chemical Co., St. Louis, MO, USA) were used to overlay the reaction mixture. PCR was conducted in a thermocycler (MiniCycler PTC 150, MJ Research Inc., Watertown, MA, USA) for 30 cycles. Each PCR cycle consisted of 45 s at 95 °C, 45 s at 54 °C and 60 s at 72 °C. The last cycle was followed by a final incubation at 72 for 3 min and cooled to 4 ºC. The polymerase chain reaction products were 602 bp (TLR2), 548 bp (TLR4) and 340 bp (GAPDH). RT-PCR products were electrophoresed on a 1.5% ethidium bromidestained agarose gel and saved as digital images. Relative quantities of TLR2 and TLR4 mRNA expression were analyzed by Bandleader 3.0 software (Magnitec Ltd., Israel), normalized with GAPDH expression.

Survival Study

Additional six groups of rats (20 rats/group) received identical treatments as mentioned above. Survival of rats was monitored for 7 days.

Statistic analysis

Data were presented as mean ± standard deviation (S.D.). Statistical Product for the Social Sciences-11.0 (SPSS Inc., Chicago, IL, USA) was

used for data analysis. Differences among groups were determined by one-way analysis of variance (ANOVA), followed by the Student-Newman-Keuls (SNK) post hoc test. Kaplan-Meier analysis was used to compare survival rates. Significance was defined as $p < 0.05$.

RESULTS

Pulmonary levels of TNF-α and IL-6

The levels of TNF-α and IL-6 in the lung tissue were elevated after CLP, compared with the sham control ($P<0.05$). Ketamine at the doses of 5 mg/kg and 10 mg/kg suppressed TNF-α and IL-6 elevation after experimental CLP ($P<0.05$). Ketamine did not influence pulmonary levels of TNF-α and IL-6 in the rats received no CLP (Fig. 1 and Fig. 2).

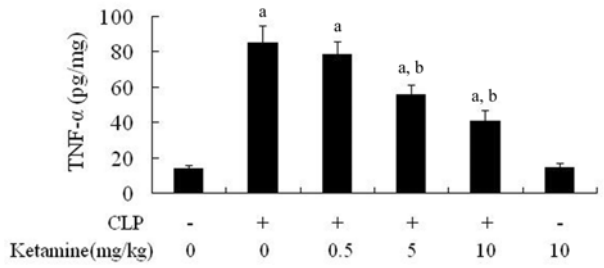

Figure 1. Production of TNF-α in rat lungs. CLP induced elevation of TNF-α, and ketamine after CLP decreased the level of TNF-α. a: $P<0.05$ versus sham control; b: $P<0.05$ versus CLP group.

Survival Study

In sham-operation group and ketamine-alone group, no rats died throughout the experiment. Survival following CLP decreased significantly ($P<0.05$). Ketamine (5 mg/kg or 10 mg/kg) led to a marked increase in survival after CLP operation ($P<0.05$) (Fig. 6).

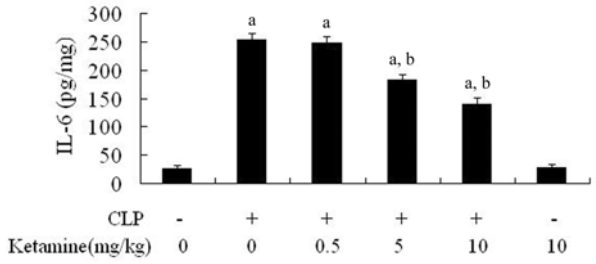

Figure 2. Production of IL-6 in rat lungs. IL-6 production was enhanced after CLP, and ketamine decreased this enhancement. a: $P<0.05$ versus sham control; b: $P<0.05$ versus CLP group.

Pulmonary NF-κB activity

CLP induced a significant increase in NF-κB activity of rat lungs ($P<0.05$). At the dose of 5 mg/kg and 10 mg/kg, ketamine inhibited the elevation of NF-κB activity after CLP ($P<0.05$). Ketamine alone did not change NF-κB activity, compared with the controls (Fig. 3).

Pulmonary TLR2 and TLR4 expression

TLR2 and TLR4 mRNA expression was statistically increased in the lungs of rats challenged with CLP, compared with those in control group ($P<0.05$). Ketamine inhibited pulmonary TLR2 expression of the CLP rats in a dose-related manner. The minimal dosage of ketamine required to suppress the CLP-induced TLR2 elevation was 5mg/kg ($P<0.05$). And ketamine 0.5, 5 and 10 mg/kg inhibited TLR4 expression after CLP ($P<0.05$). Ketamine alone had no effect on pulmonary levels of TLR2 and TLR4 in rats without CLP (Fig. 4 and Fig. 5).

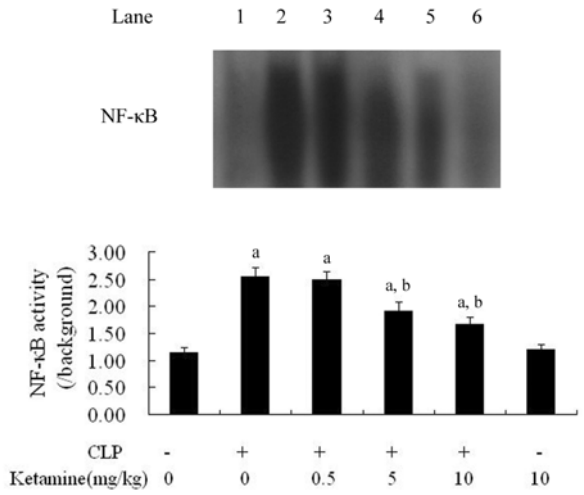

Figure 3. Activity of NF-κB in rat lungs. Lane 1, Sham-operation control group; Lane 2, CLP group; Lane 3, CLP+ketamine (0.5 mg/kg) group; Lane 4, CLP+ketamine (5 mg/kg) group; Lane 5, CLP+ketamine (10 mg/kg) group; Lane 6, Ketamine (10 mg/kg) alone group. CLP increased pulmonary NF-κB activity, and ketamine suppressed this effect. a: P<0.05 versus sham control; b: P<0.05 versus CLP group.

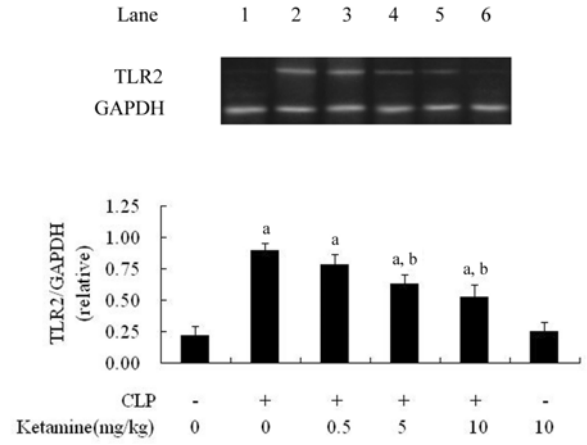

Figure 4. TLR2 expression in rat lungs. Lane 1, Sham-operation control group; Lane 2, CLP group; Lane 3, CLP+ketamine (0.5 mg/kg) group; Lane 4, CLP+ketamine (5 mg/kg) group; Lane 5, CLP+ketamine (10 mg/kg) group; Lane 6, Ketamine (10 mg/kg) alone group.CLP enhanced pulmonary TLR2 mRNA expression, and ketamine after CLP inhibited this effect. a: P<0.05 versus sham control; b: P<0.05 versus CLP group.

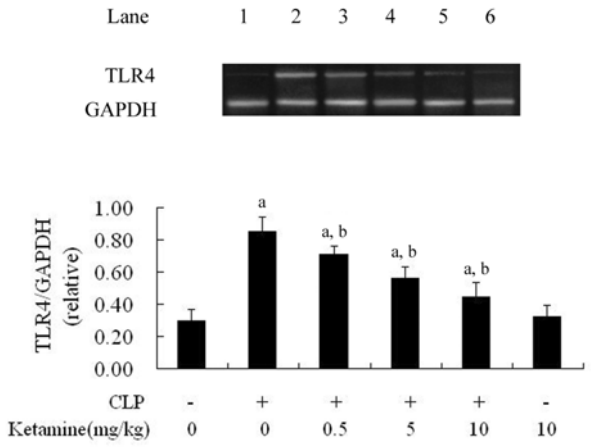

Figure 5. TLR4 expression in rat lungs. Lane 1, Sham-operation control group; Lane 2, CLP group; Lane 3, CLP+ketamine (0.5 mg/kg) group; Lane 4, CLP+ketamine (5 mg/kg) group; Lane 5, CLP+ketamine (10 mg/kg) group; Lane 6, Ketamine (10 mg/kg) alone group. Expression of TLR4 mRNA was elevated after CLP, and ketamine inhibited this elevation. a: P<0.05 versus sham control; b: P<0.05 versus CLP group.

DISCUSSION

Our study demonstrated that the CLP operation increased pulmonary levels of TNF-α, IL-6, NF-κB, TLR2 and TLR4 of rats. The results also indicated that, during CLP-induced sepsis, ketamine could suppress the production of TNF-α and IL-6 and inhibit the activation of NF-κB in rat lungs. Moreover, ketamine suppressed the pulmonary TLR2 and TLR4 expression of septic rats in a dose-related manner. In addition, ketamine after CLP may improve the survival of rats.

TNF-α holds a key position in the inflammatory responses during sepsis (19). It is believed that TNF-α is one of the primary agents which may initiate the cytokine cascade in sepsis, and may cause systemic inflammatory response syndrome (SIRS) (20). In addition to TNF-α, IL-6 is also a notable element in the cytokine network during sepsis.

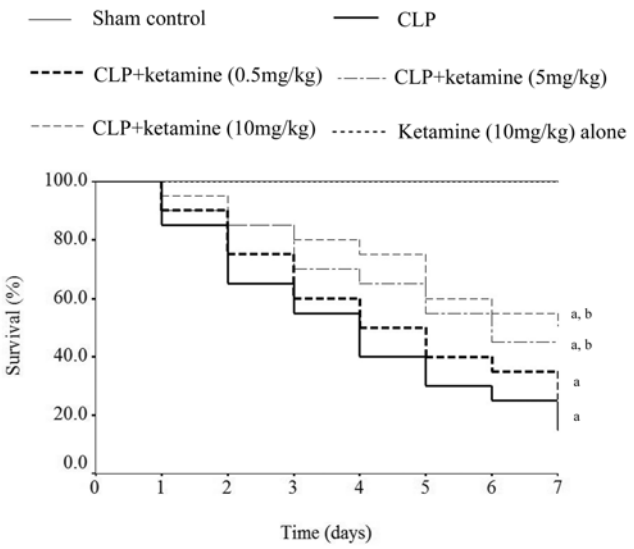

Figure 6. Kaplan-Meier curves for survival of rats. CLP decreased survival of the rats, and ketamine after CLP increased survival. a: P<0.05 versus sham control; b: P<0.05 versus CLP group.

Previous studies indicated that IL-6 concentration often closely correlats with the severity of sepsis (21-22). In the present study, we investigated the effects of ketamine on pulmonary levels of TNF-α and IL-6, both of which are powerful inflammatory cytokines, in the CLP model of sepsis. Our study indicated that ketamine could suppress the CLP-induced elevation of TNF-α and IL-6 production. These findings corresponded well with previous reports that ketamine could inhibit the production of TNF-α and IL-6 after LPS stimulation (5-11). Moreover, in the CLP model of sepsis, we found that the minimal dose of ketamine to suppress TNF-α and IL-6 in rat lungs was 5 mg/kg. However, a previous report implicated that ketamine 0.25 mg/kg could attenuate the increase of serum IL-6 of patients after cardiopulmonary bypass (CPB) (23). There might be two reasons for the difference in the doses of ketamine to suppress IL-6. First, the models are different. The impairment of CLP may be different from the injury of CPB. Second, there is difference in the objects. The dose of ketamine required to suppress the production of IL-6 in rat lungs may be different from that in sera of human beings.

The transcription factor NF-κB is believed to regulate the expression of a wide variety of genes which are essential in innate immune responses, including those encoding cytokines such as TNF-α and IL-6 (8). Persistent activation of NF-κB may lead to excessive production of inflammatory cytokines, culminating in tissue injury, organ dysfunction, or even death. In the present study, we performed EMSA to examine the NF-κB activity. We found that the NF-κB activity in rat lungs was elevated after CLP operation. Previous work from our laboratory and others indicated that ketamine could inhibit NF-κB activation after LPS stimulation in vivo and in vitro (6, 9, 24). In agreement with previous reports, the present study also revealed that ketamine could suppress NF-κB activation in rat lungs during CLP-induced polymicrobial sepsis. This result was consistent with the influence of ketamine on TNF-α and IL-6 after CLP.

The Toll-like receptors (TLRs) have been highlighted recently in pathogen recognition and host defense. (25) These TLRs are capable of recognizing and discriminating diverse pathogen-associated molecular patterns (PAMPs). In the TLRs family, TLR2 and TLR4 have been extensively studied. In mammals, TLR2 seems to be involved in recognition of bacterial lipoprotein (BLP), peptidoglycan (PGN), lipoteichoic acid (LTA) of Gram-positive bacteria, and lipoarabinomannan (LAM) of mycobacteria and mannans of yeasts (26-27), while TLR4 may recognize LPS of Gram-negative bacteria (27-29). It has been suggested that TLR2 and TLR4 may transduce the signals from the extracellular domain to the cytoplasm, leading to the activation of immune responses, especially the activation of NF-κB (16). In our study, we used the model of

CLP-induced sepsis, which is composed of both Gram-positive and Gram-negative bacteremia (30), to investigate the effect of ketamine on TLR2 and TLR4 expression in rat lungs. Our study revealed that the pulmonary levels of TLR2 and TLR4 increased after CLP operation. This result was in agreement with previous reports that TLR4 expression was up-regulated in cultured rat lung pericytes and in rat lungs after LPS stimulation (31, 32). The present investigation also indicated that ketamine after CLP suppressed TLR2 and TLR4 expression in rat lungs. The influence of ketamine on TLR2 and TLR4 was consistent with its effect on NF-κB, as well as TNF-α and IL-6.

Ketamine has been reported to suppress LPS-induced mortality in carrageenan-sensitized rats (10). It also has been demonstrated that ketamine could improve survival in burn injury followed by sepsis in rats (33). In the present study, we found that ketamine at the doses of 5 mg/kg and 10 mg/kg could improve survival in a CLP model of sepsis. Previous studies suggested that the beneficial effect of ketamine on survival was probably achieved by attenuating production of inflammatory cytokines (10, 33). Moreover, it was implied that inhibiting NF-κB activation and TLR2 and TLR4 expression correlated with survival improvement in sepsis (34, 35). Therefore, we hypothesized that, in the CLP model of sepsis, ketamine might improve survival by suppressing production of inflammatory cytokines, NF-κB activation and TLR2 and TLR4 expression. The results of present study supported our hypothesis, which led us to conclude that, during sepsis induced by CLP, the beneficial effect of ketamine on the survival of rats possibly correlates with its anti-inflammatory effects.

In this study, we used ketamine at the dosage levels of 0.5 mg/kg, 5 mg/kg and 10 mg/kg. Our results revealed that ketamine at these doses inhibited TLR4 expression in CLP-induced sepsis. At 5 mg/kg and 10 mg/kg, ketamine after CLP suppressed TNF-α and IL-6 production, NF-κB activity and TLR2 expression in rat lungs, and improved the survival of rats. The doses of ketamine 5 mg/kg and 10 mg/kg seem to be relatively high compared with the doses usually used in anesthesia of humans. However, there may be differences in the doses of ketamine between humans and animals. It has been suggested that the doses of ketamine required to anesthetize the experiment animal such as guinea-pig range from 44 to 250 mg/kg (36). And according to previous reports, no anesthetic state was produced in rats received 10 mg/kg ketamine (37). So the doses of ketamine in our study may be sub-anesthetic to rats.

In conclusion, our study indicated that in the CLP model of sepsis, ketamine at sub-anesthetic doses could suppress inflammatory cytokine production, NF-κB activation, and TLR2 and TLR4 expression. And these anti-inflammatory effects of ketamine possibly correlate with improved survival of rats with CLP-induced sepsis. These findings suggest that proper use of ketamine may offer advantages during sepsis.

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