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Etomidate inhibits nuclear factor-κB through decreased expression of glucocorticoid receptor in septic rats

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Abstract. The present study aimed to investigate the effect of etomidate administered prior to or following cecal ligation and puncture (CLP) on the expression of glucocorticoid receptor (GR) and lymphocyte apoptosis in septic rats. Right jugular vein catheterization was performed on female Sprague-Dawley rats under isoflurane anesthesia, and CLP surgery was performed to induce sepsis 3 days following catheterization. The rats were randomly divided into five groups. All groups were infused with 2 ml of either etomidate or 5‰ dimethyl sulfoxide (DMSO) at 1 ml/h for 2 h from 6 h post-surgery. The sham group received abdominal sham surgery and infusion with DMSO; the CLP control group received infusion with DMSO. Treatment group A received infusion with 2 mg/kg etomidate; group B received 0.6 mg/kg etomidate following CLP and an infusion of 2 mg/kg etomidate. Group C received 0.6 mg/kg etomidate 24 h prior to CLP and post-surgical etomidate infusion. The 10-day survival rates of the rats in the CLP, A, B and C groups were 60, 50, 55 and 40%, respectively. The serum mRNA expression levels of tumor necrosis factor-α, GR and glucocorticoid-induced leucine zipper were detected by reverse transcription-quantitative polymerase chain reaction, the abundance of inhibitor of nuclear factor (NF)-κB-α was measured by western blotting, and the apoptotic rates of the splenic lymphocytes were determined using flow cytometry. The results suggested that etomidate inhibited NF-κB by decreasing the expression of GR in the septic rats. The increased apoptosis of lymphocytes induced by etomidate may lead to a poor outcome during sepsis.

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Introduction

Sepsis remains a challenge in critical patients due to the potentially life-threatening levels of whole-body inflammation. As a result, sepsis is a leading contributor to rates of mortality in intensive care units worldwide (1). Etomidate is a short-acting intravenous anesthetic, which is well known for its mild repression of hemodynamics in rapid sequence intubation; however, this treatment is associated with a period of adrenal steroidogenesis suppression following injection (2). As adrenal insufficiency or low serum cortisol can lead to poor outcomes in patients with sepsis, the use of etomidate in patients with sepsis is controversial. Although numerous studies have been performed, the effect of etomidate on mortality rates in sepsis, and the underlying mechanisms responsible for its effects, remain to be fully elucidated (3-5). Previous investigation in animals suggested that etomidate increases mortality rates in septic rats, although this was not associated with adrenal insufficiency (6). The present study aimed to investigate the glucocorticoid-associated anti-inflammatory effect of etomidate, the apoptosis of lymphocytes and the survival rates of septic rats following treatment.

Materials and methods

Animals and experimental protocol. Adult female Sprague-Dawley rats (210-240 g) were purchased from Dalian Medical University (Dalian, China). The rats (n=25/group) were acclimated under laboratory conditions (12 h light/dark cycle; 20-24˚C) for 1 week prior to experimentation. Water and food were provided *ad libitum* throughout the experiment. Animal experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD, USA), and protocols were approved by the Animal Care and Use Committee of Dalian Medical University (permit no. 20140708-5). All surgical procedures were performed using aseptic techniques. To enable continuous injection, right jugular vein catheterization was performed on the rats under 2-4% isoflurane anesthesia prior to the start of the experiment protocols. The catheterization procedures were performed according to instructions previously reported in the literature (7). Following a recovery period of 3 days, all rats, with the exception of those in the sham group, underwent surgery for

cecal ligation and puncture (CLP) under isoflurane anesthesia. The CLP was performed according to previously described techniques (8). Briefly, the rats were anesthetized by inhalation of 1-2% isoflurane through a nose cone. Subsequently, a 2-cm midline abdominal incision was made, and one-third of the distal cecum was ligated and penetrated twice, crosswise, with a 21-gauge needle, which led to the induction of sepsis. Following this, the cecum was returned and the abdomen was closed. The rats were injected with 1 ml normal saline with 0.25 μg/ml sufentanil (Yichang Renfu Pharmaceutical Co., Ltd., Hubei, China) subcutaneously for postoperative analgesia. The rats were randomly assigned into one of five groups (Fig. 1). All groups were infused with 2 ml of either etomidate (Enhua Pharmaceutical, Jiangsu, China) or 5% dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) solution at 1 ml/h for 2 h from 6 h post-surgery. The sham group, in which a 2-cm vertical incision was made on the midline of the abdomen prior to suturing, was infused with 5% DMSO 6 h post-surgery using a micro injection pump (BD Biosciences, San Jose, CA, USA) through the right jugular vein catheter. The CLP group was administered with a 5% DMSO infusion 6 h post-CLP surgery. Treatment group A was administered with a 2-h infusion of 2 mg/kg/h etomidate dissolved in 5% DMSO solution 6 h post-CLP surgery; group B was administered with a 1-m bolus injection of etomidate (0.6 mg/kg) at the time of CLP, followed by a 2-h infusion of 2 mg/kg/h etomidate (as in group A); and group C was administered with 1 ml of etomidate (0.6 mg/kg) 24 h prior to CLP, followed by a 2-h etomidate infusion. Due to the circadian rhythm associated with corticosterone (CORT), the surgery was performed between 8:00 and 9:00 a.m. The rats were sacrificed by inhaled overdose of isoflurane (6-8%, 5 min) 24 h following surgery, subsequent to which bilateral adrenal glands and an arterial blood sample were collected. As previous studies have suggested that female mice are more resistant to CLP, compared with male mice (9), female rats were selected in the present study to avoid gender-associated genetic differences in response to sepsis. Arterial blood samples were collected 24 h following CLP surgery; the bilateral adrenal glands and spleens were collected immediately following the sacrifice of the rats (n=5 per group). Additional rats (n=20 per group), with the exception of the sham group, were observed for 10 days to monitor survival rates following surgery. The conditions of the animals were assessed at 6:00 a.m and 6:00 p.m. each day.

Enzyme-linked immunosorbent assay. The serum levels of tumor necrosis factor-α (TNF-α) and CORT were measured using a rat ELISA kit (Cusabio, Wuhan, China) according to the manufacturer's protocol. The minimal detection levels of TNF-α and CORT were 6.5 and 0.2 pg/ml, respectively.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA from the adrenal gland was isolated using the RNA prep pure tissue kit (Tiangen Biotech Co., Ltd., Beijing, China). Total RNA (~90 μg) was reverse-transcribed using the PrimeScript RT Mix kit (Takara Biotechnology Co., Ltd., Dalian, China). The following primers were used: Glucocorticoid receptor (GR), forward 5'-CCGCAGTAGCAGGGTTATTTTC-3' and reverse 5'-GAAGGGTGGGGAGGATTAGTGT-3'; glucocorticoid-induced leucine zipper (GILZ), forward 5'-TGGAATGCCAATATGCTCCAG-3' and reverse 5'-AGGAACAGTCGTTGTCAGGTGAA-3'; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5'-GGCACAGTCAAGGCTGAGAATG-3' and reverse 5'-ATGGTGGTGAAGACGCCAGTA-3'. A total of 100 ng complementary DNA was added to a 20-μl reaction for qPCR analysis with SYBR premix Ex Taq II (Takara Biotechnology Co., Ltd.) and then amplified using the LightCycler 480 automatic PCR and analysis system (Roche Applied Science, Indianapolis, IN, USA). GAPDH was used as an internal control gene. The comparative quantification (CQ) method was used to calculate the relative expression of the target gene (10). The qPCR analysis was performed under the following conditions: Denaturation for 30 sec at 95˚C, followed by 40 cycles of denaturation for 5 sec at 95˚C, annealing for 30 sec at 60˚C and cooling for 30 sec at 50˚C.

Protein extraction and western blot analysis. The adrenal glands were homogenized in lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), and tissue proteins were extracted using a protein extraction kit (Beyotime Institute of Biotechnology). The Bradford method was used to determine the protein concentrations. Equal quantities of the protein samples (~60 μg) from each group were resolved on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate, and then transferred onto Immobilon-P PVDF membranes (Merck Millipore). After blocking with 5% milk in TBS for 1 h at room temperature, the membranes were probed with rabbit anti-mouse primary antibodies against inhibitor of nuclear factor (NF)-κB (IκB-α; 1:500; cat. no. 10268-1-AP; Proteintech Group, Inc., Chicago, IL, USA) and tubulin-α (1:500; cat. no. 11224-1-AP; Proteintech Group, Inc.) at 4˚C overnight. Subsequently, the membranes were washed extensively with TBS-0.1% Tween-20 (TBST) and probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:5,000; cat. no. ab6721; Abcam, Cambridge, UK) conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. Following washing with TBST, the immunoreactive bands were visualized using Luminata Classico Western HRP substrate (Merck Millipore). Tubulin-α was used as a loading control to normalize IκB-α. The band intensities were quantified using Quantity One software (version 4.6.2 for Windows; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Examination of the apoptotic rate of lymphocytes in the spleen. Lymphocytes were obtained from fresh rat spleens. Briefly, fresh spleens were isolated and cut into small sections. Subsequently, several of these sections were mechanically disrupted on a nylon mesh in a 35-mm dish containing 4-5 ml lymphocyte separation liquid (Dakewe Biotech Co., Ltd., Shenzhen, China). The suspension was transferred to a 15-ml centrifuge tube containing 200-500 μl RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and then centrifuged at ~108 x *g* for 30 min. Finally, the lymphocytes were aspirated and washed twice with phosphate-buffered saline (pH 7.4; Merck Millipore), following which the cell density was calculated.

Following adjustment of the cell density to $2x10^6$/ml per sample, a cell apoptosis assay kit (Biouniquer, Nanjing, China), including fluorescein isothiocyanate-labeled annexin V and

propidium iodide, was used to determine the apoptotic rate with a flow cytometer (FACS Calibur; BD Biosciences).

Statistical analysis. The Kolmogorov-Smirnov test was performed to examine the normality of the data. The quantitative data, which passed normality assessment, are presented as the mean ± standard error of the mean. One-way analysis of variance followed by Tukey's test was performed for comparisons among the groups. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank (Mantel-Cox) test. GraphPad Prism (version 6.0 for Mac; GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze the data and generate histograms. $P<0.05$ was considered to indicate a statistically significant difference.

Results

The different etomidate treatment protocols led to different survival rates (Fig. 2), which suggested that etomidate had effects on targets other than the adrenal glands during sepsis. The survival rate in group C was 40%, which was lower, compared with that in the CLP group (60%). However, no significant difference was found between the treatment groups. High levels of inflammatory cytokines released during the early phase of sepsis led to a series of systematic inflammatory responses throughout the body. TNF-α is one such inflammatory cytokine, and the level of serum TNF-α can be used to assess the extent of inflammation. Etomidate treatment inhibited the serum level of TNF-α (Fig. 3A), which suggested that inflammation was inhibited in the treated septic rats. Although it is well known that etomidate inhibits adrenal steroidogenesis by inhibiting the activity of 11β-hydroxylase, which coverts 11-deoxycorticosterone to CORT, the serum levels of CORT in the control and treated groups were not markedly decreased. However, the level of CORT in group C was significantly lower, compared with the levels in the other groups (Fig. 3B). This result suggested that the inhibition of etomidate-induced steroidogenesis may not have affected the serum levels of CORT over the short period of time, or that this inhibition may have a delayed effect on the levels of CORT. The majority of CORT binds to GRs, and the observed decrease in the mRNA expression level of GR suggested that etomidate may have inhibited the expression of GR, either directly or indirectly. However, pretreatment with etomidate (group C) appeared to alleviate the decline in the mRNA expression of GR (Fig. 3C). The mRNA expression of GILZ, an important mediator of glucocorticoid action, was in accordance with that of GR (Fig. 3D), which further suggested that etomidate may have inhibited the GR signaling pathway. Furthermore, when NF-κB was activated, IκB-α was ubiquitinated in the cytoplasm and its expression level decreased. The expression level of IκB-α in groups A, B and C were significantly higher, compared with that observed in the CLP group (Fig. 4). Taken together, the results presented above suggested that etomidate may inhibit NF-κB activation through the GR signaling pathway. However, the increased apoptotic rate of the lymphocytes obtained from groups B and C suggested that etomidate treatment stimulated lymphocyte apoptosis, which may harm the immune system and suppress the immune response (Fig. 5).

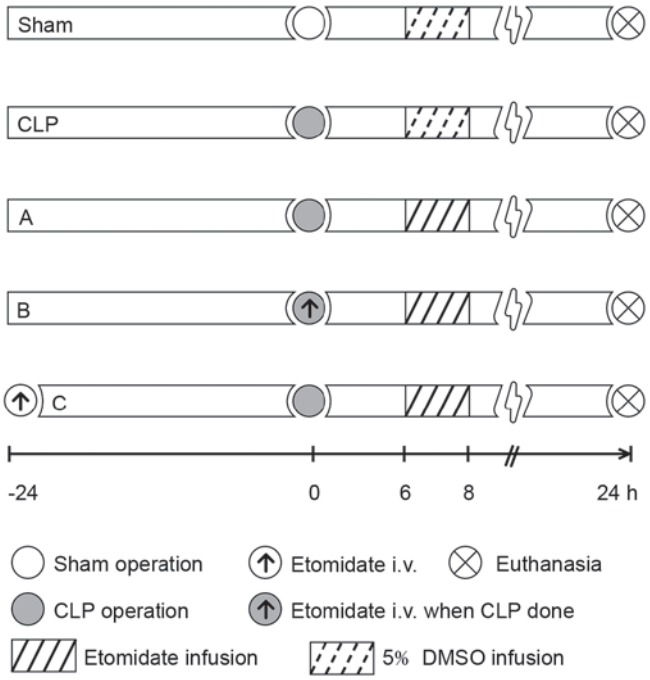

Figure 1. Schematic diagram of the etomidate treatment protocol in the Sham, CLP, A, B and C treatment groups. CLP, cecal ligation and puncture; DMSO, dimethyl sulfoxide.

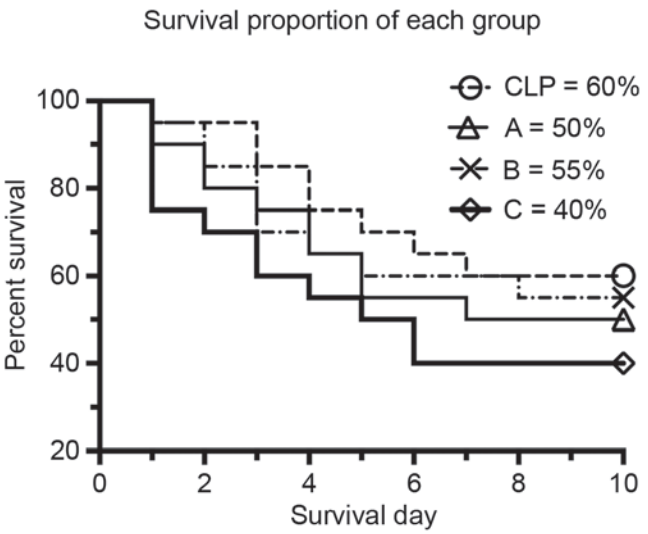

Figure 2. Kaplan-Meier survival curve of the percentage survival over 10 days (n=20). The survival rates in the CLP, A, B and C groups were 60, 50, 55 and 40%, respectively. CLP, cecal ligation and puncture.

Discussion

Advantages and disadvantages of animal models. Right jugular vein catheterization and placement of the catheter at the back of the rat's neck enable intravenous injections following surgery and infusion for a relatively long duration. The injection of a 0.6 mg/kg bolus of etomidate and a 2-h infusion of 2 mg/kg/h etomidate can cause significant sedation without respiratory depression in rats. The most commonly used animal models of sepsis are the endotoxemia model, the bacterial-inoculum model and the CLP model (11). The

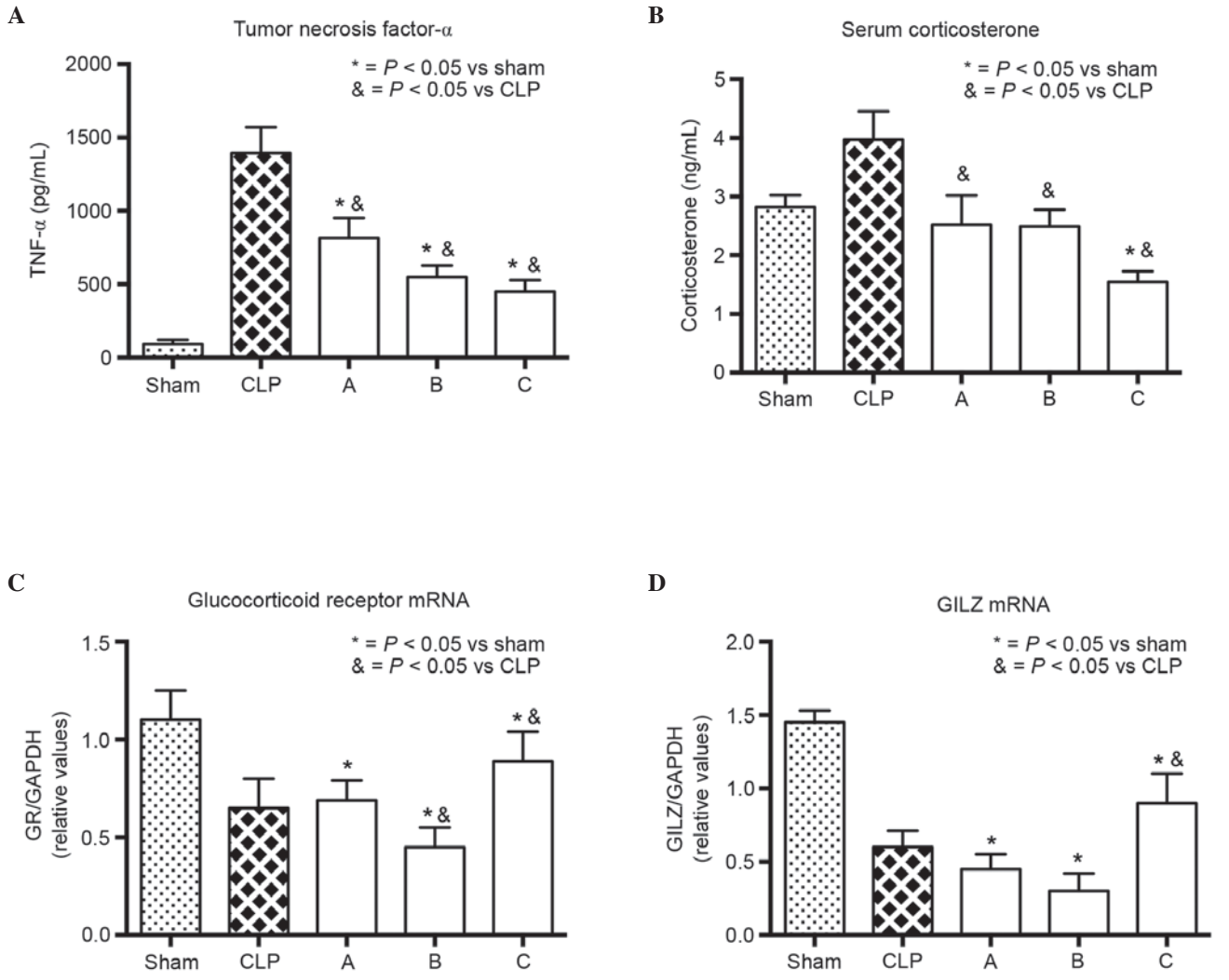

Figure 3. Histograms of the levels of TNF-α, CORT, GR and GILZ. (A) Serum levels of TNF-α of the rats in each group (n=5). (B) Serum levels of CORT in each group (n=5). (C) mRNA expression levels of GR in the adrenal glands of the rats in each group (n=5). (D) mRNA expression levels of GILZ in the adrenal glands of the rats in each group (n=5). Values are presented as the mean + standard error of the mean. *P<0.05, compared with the sham group; &P<0.05, compared with the CLP group. CLP, cecal ligation and puncture; TNF-α; tumor necrosis factor-α; CORT, corticosterone; GR glucocorticoid receptor; GILZ, glucocorticoid-induced leucine zipper; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

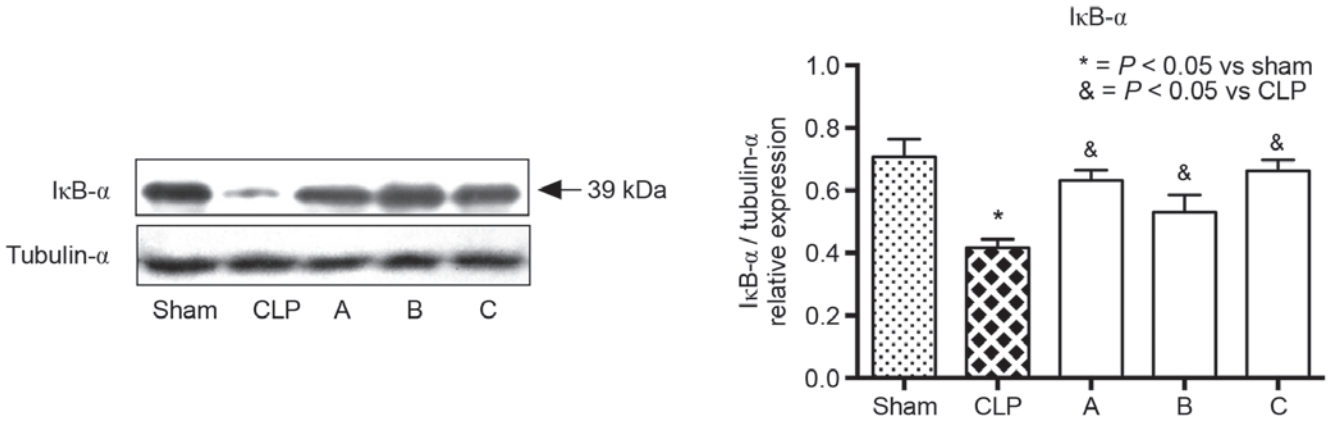

Figure 4. Expression of IκB-α in the adrenal glands of each group. Representative blots for the IκB-α proteins are shown above the histogram. As the activation of NF-κB was initiated by the signal-induced ubiquitination of IκB-α proteins, the decreased abundance of IκB-α proteins in the CLP group suggested NF-κB had been activated. The expression levels of IκB-α in treatment groups A, B, and C were significantly higher, compared with that in the CLP group, which suggested that etomidate treatment inhibited NF-κB activity. Values are presented as the mean + standard error of the mean (n=5). *P<0.05, compared with the sham group; &P<0.05, compared with the CLP group. CLP, cecal ligation and puncture; NF-κB, nuclear factor-κB; IκBα, inhibitor of NF-κB-α.

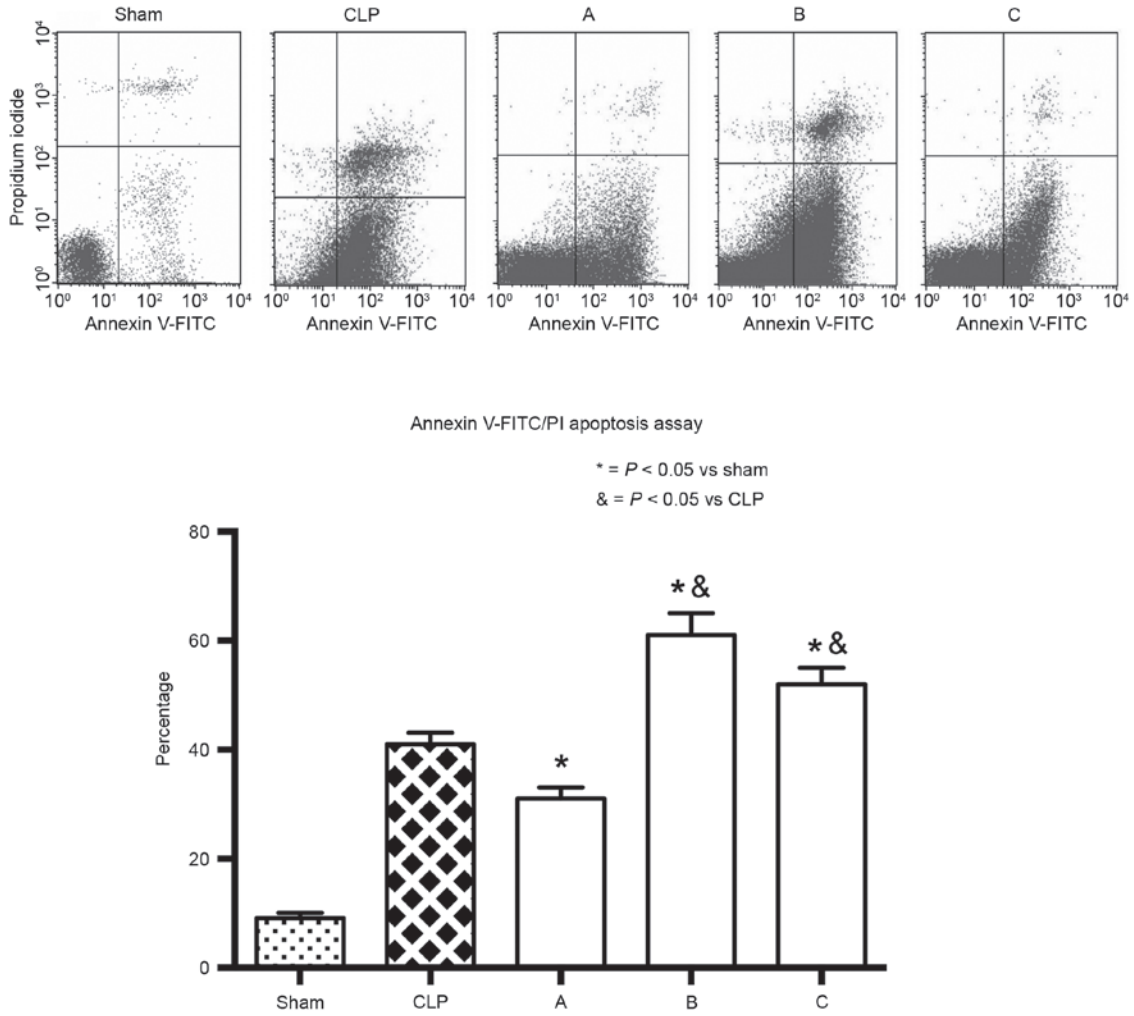

Figure 5. Apoptotic rates of splenic lymphocytes in each group. The apoptotic rates were assessed using flow cytometry with Annexin-V/propidium iodide staining. Representative computational dot plots of the results of the flow cytometry for each group are shown above the histogram. Values are presented as the mean + standard error of the mean (n=5). *P<0.05, compared with the sham group; &P<0.05, compared with the CLP group. CLP, cecal ligation and puncture; FITC, fluorescein isothiocyanate.

administration of lipopolysaccharide (LPS) is one of the most common strategies to induce sepsis in the endotoxemia model. Although this model is controlled and standardized, the rapid, transient and waterfall-like cytokine responses following LPS injection differ from those observed in human sepsis. Thus, LPS injection may not be suitable for the investigation of anesthetics during sepsis over a longer time period. The bacterial inoculum model requires bacterial growth and quantification prior to administration. By contrast, the CLP model is a polymicrobial sepsis model, which is readily reproducible and induces a prolonged, but not excessively high, elevation of cytokine levels, as observed in humans (11). However, the limitation of the CLP model is the variability in severity due to differences between surgical procedures. The present study standardized the key parameters of CLP surgery, including the length of ligation, the gauge of the puncture needle and identical post-operative recovery conditions, to increase the sample size but minimize variability.

Anti-inflammatory effect of etomidate and survival rates of treated rats. In the present study, the decreased levels of TNF-α in groups A, B and C demonstrated the anti-inflammatory effect of etomidate. Previously, anesthetics and sedatives other than etomidate have been shown to have anti-inflammatory effects and to inhibit the activity of NF-κB, including propofol (12), dexmedetomidine (13) and midazolam (14). However, the survival data in the present study showed that, when etomidate was administered 24 h prior to surgery, survival rates decreased to 40%, which was lower, compared with the rate of 60% observed in the CLP group. Therefore, it appeared that the anti-inflammatory effects of etomidate may not have improved the outcome of sepsis. Glucocorticoids are a natural anti-inflammatory agent in the body, and serum levels of CORT increase to resist inflammation in the presence of a functioning hypothalamic-pituitary-adrenal axis (15). In the present study, the levels of CORT were lowest in group C, which made it difficult to conclude that etomidate induced adrenal insufficiency in sepsis. Considering that the normal range of CORT levels in humans fluctuates between 55 and 635 nmol/l from day to night, a gold standard is required for the diagnosis of relative adrenal insufficiency (16). It may be that the decrease in etomidate-induced CORT occurs 24 h following injection, as it takes time for etomidate to inhibit the activity of 11β-hydroxylase. Previous studies (17,18) have

revealed that adrenal suppression persists for >24 h following etomidate infusion. However, considering the mRNA expression levels of GR and GILZ observed in the present study, it is possible that etomidate may have also inhibited the expression of GR in the adrenal gland. Virtually all the effects of CORT are mediated by the activation of the GR. According to the commonly accepted theory, GR is a transcription factor, which exists in its inactive form in the cytoplasm. Once activated by binding to CORT, the CORT-GR complex homodimerizes and then migrates to the nucleus, where it binds to specific DNA to modulate cell function via protein synthesis (19,20). GILZ, which is upregulated by GR, is a mediator of the anti-inflammatory effects of CORT. GILZ is also a promising candidate as a therapeutic anti-inflammatory drug as it has no detrimental effect of glucocorticoids (21). In the present study, the mRNA expression levels of GR and GILZ were similar among the groups. In addition, the levels of IκB in the adrenal gland were not decreased as expected, which suggested that etomidate also inhibited the translocation of NF-κB. A previous study (6) observed that etomidate reduces the level of inflammatory cytokines by inhibiting the activation of NF-κB. Other studies have reported that the crosstalk between glucocorticoids and NF-κB results in the inhibition of NF-κB activity (22), and that this inhibition can be ascribed to the increased synthesis of IκB (23). Thus, according to the expression levels of CORT, GR, GILZ and IκB in the present study, it was hypothesized that etomidate inhibited the GR signaling pathway and then inhibited the NF-κB pathway during sepsis.

Effect of etomidate on lymphocytes. The traditional therapeutic strategies for sepsis have focused on the uncontrolled inflammatory response, which has led to attempts to inhibit mediators of inflammation, including LPS and TNF-α. However, these strategies (24) have largely failed. As a result, a novel paradigm has emerged, which is focused on the biphasic immune response of sepsis, with an initial hyper-inflammatory phase followed by an immunoparalysis phase. Of note, the immunoparalysis phase often determines patient survival rate, as this is a vulnerable period when patients are at risk of secondary infection from invading pathogens. The mechanism for immune paralysis appears to involve the apoptosis of immune cells, particularly lymphocytes. Weber *et al* (25) demonstrated that circulating lymphocytes in patients with severe sepsis showed accelerated apoptosis. In addition, Muenzer *et al* (26) found that the improved survival rates of CLP-induced septic mice were associated with decreased lymphocyte apoptosis. Therefore, modulating lymphocyte apoptosis has increasingly been considered to be an important stage in sepsis. Another previous study (6) observed a higher mortality rate in the etomidate pretreatment group; groups B and C showed a higher lymphocyte apoptotic rate, compared with the CLP control and sham groups. In addition, Payen *et al* (27) found that critically ill patients do not benefit from hydrocortisone used to treat etomidate-induced adrenal insufficiency. Thus, it is possible that the detrimental effects of etomidate in sepsis may be ascribed to its effect on immune suppression rather than adrenal CORT suppression, which assists in explaining why glucocorticoid supplementary therapy may not be effective in patients with sepsis.

Limitations. The present study had several limitations. First, GR protein and the CORT-GR complex are known to translocate from the cytoplasm to the nucleus following the activation of GR; thus, if the quantity of GR protein in the cytoplasm and the nucleus had been determined in the present study, the results may have been more accurate and convincing. Second, GR protein and activity are altered during the different phases of sepsis; thus, the inclusion of additional time points may assist in explaining how the trend of CORT and GR is affected by etomidate in the hyper-inflammatory and immunosuppression phases. Finally, the present study only focused on the expression of adrenal GR. Kanczkowski *et al* (28) found that immune cells are important in adrenocortical and adrenal inflammation in sepsis. Therefore, investigating the expression of GR-associated genes and proteins in immune cells may provide additional insights. A previous study (29) reported that benzodiazepine, a sedative similar to etomidate, augments γ-amino-butyric acid, suggesting that it may increase mortality rates in mice with pneumonia. Therefore, the detrimental effect of etomidate in sepsis may not be due to its inhibition of CORT alone; etomidate may also inhibit other receptors in immune cells, the central nervous system and other systems of the human body.

The results of the present study indicated that etomidate treatment may alleviate inflammation in the adrenal gland in sepsis through the inhibition of GR and NF-κB translocation. However, etomidate stimulated the apoptosis of lymphocytes, which may lead to poor outcomes in sepsis, although additional evidence is required to investigate this effect. According to the results of the present study, anesthesiologists may be able to safely use etomidate once for induction in critical patients. However, overdose or the repeated use of etomidate in patients with sepsis may be harmful due to the delayed or accumulated immunosuppression induced by etomidate. By investigating etomidate-induced immunosuppression, a more comprehensive understanding of the immunomodulation and endocrinomodulation in sepsis can be obtained, and novel therapeutic strategies to treat sepsis may be identified in the future.

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