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Early Administration of Cisatracurium Attenuates Sepsis-Induced Diaphragm Dysfunction in Rats

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Abstract—Sepsis can often induce diaphragm dysfunction, which is associated with localized elaboration of cytokines within the diaphragm. The administration of cisatracurium has been shown to decrease the inflammatory response and to facilitate mechanical ventilation. In this study, we explored whether cisatracurium could attenuate sepsis-induced diaphragm dysfunction in rats. Animals were divided into three groups: (1) the control group: rats underwent a sham surgical procedure with cecal exposure, but the cecum was neither ligated nor punctured; (2) the CLP group: rats underwent cecal ligation and puncture (CLP) and received a continuous infusion of NaCl 0.9 %; and (3) the Cis+CLP group: rats underwent CLP and received a continuous infusion of cisatracurium. After the surgical procedure, all animals underwent controlled mechanical ventilation for 18 h. Plasma concentrations of interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and high-mobility group box 1 (HMGB1) were measured using an enzyme-linked immunosorbent assay. Upon completion of the experimental protocol, diaphragm contractility and HMGB1 protein expression were analyzed. Impaired diaphragm contractile function, including both force-related properties and force-frequency responses, was pronounced after CLP in comparison with that observed in the control rats. Furthermore, CLP elevated serum levels of IL-6, TNF-α, and HMGB1, and induced HMGB1 protein expression in the diaphragm. In contrast, cisatracurium counteracted the sepsis-induced inflammation reaction in the diaphragm and serum and maintained diaphragm function. These data suggest that early infusion of cisatracurium attenuates sepsis-induced diaphragm dysfunction; this may be attributable to its anti-inflammatory action.

KEY WORDS: sepsis; diaphragm dysfunction; high-mobility group box 1; cisatracurium.

INTRODUCTION

Severe sepsis elicits multiple organ system failure, and respiratory muscles are vulnerable to sepsis. Increasing clinical evidence indicates that 70–100 % of patients with severe sepsis required prolonged mechanical ventilation (MV) that was strongly associated with diaphragm dysfunction [1–4]. Although multiple risk factors are involved in this process, including the localized elaboration of cytokines within the skeletal muscle, excessive free-radical generation from a number of different sources, enhanced proteolytic degradation, and decreased protein synthesis, the excessive release of pro-inflammatory cytokines during sepsis was the principal reason for deteriorated diaphragmatic function [5].

High-mobility group box 1 (HMGB1), a non-histone chromosomal protein, is a lethal inflammatory cytokine involved in sepsis [6]. In a setting of severe sepsis, this protein is delayed with respect to pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), which are released within a few hours after the onset of inflammation. Studies confirmed that HMGB1 expression is associated with diaphragmatic dysfunction in CLP animals and that the administration of anti-HMGB1 antibodies or inhibitors significantly improved survival in septic rats and reduced damage to multiple organ structures [7–9].

When controlled ventilation is used in critically ill patients, non-depolarizing neuromuscular blocking drugs

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(NMBDs) are often used to facilitate MV and improve the tissue oxygen supply. Recent research has demonstrated that the infusion of NMBDs might affect intensive care unit (ICU) stay duration. Cisatracurium (Cis), one of the most widely used NMBDs that undergo Hofmann elimination, has an organ-independent elimination. There are minor differences in the pharmacokinetics of cisatracurium in various patient populations and was recommended for patients with renal or hepatic failure and elderly patients. Several studies have shown that the administration of cisatracurium could decrease inflammation in patients with sepsis and even increase the survival rate of patients by delaying muscle weakness [10–13]. However, whether NMBDs alleviate respiratory muscle inflammation or eliminate its functional deterioration is still unknown. We hypothesized that the administration of cisatracurium in the early stages of sepsis would decrease systemic and local inflammation and would subsequently attenuate sepsis-induced diaphragmatic dysfunction.

MATERIALS AND METHODS

Animals and Groups

All experiments were approved by the Animal Care and Use Committee of the Shanghai Jiaotong University School of Medicine. A total of 30 male adult Sprague-Dawley rats (9 weeks old) were included in the experiment. The animals were maintained in 12 h/12 h light/dark cycles at an ambient temperature (23–25 °C) and provided with standard laboratory rat chow and water ad libitum.

Rats were randomly divided into three groups of ten animals each: (1) the control group: rats underwent a sham surgical procedure with cecal exposure, but the cecum was neither ligated nor punctured; (2) the CLP group: rats underwent cecal ligation and puncture (CLP) and received a continuous infusion of NaCl 0.9 %; and (3) the Cis+CLP group: rats underwent CLP and received a continuous infusion of cisatracurium. After the surgical procedure, all animals were submitted to controlled MV for 18 h.

Experimental Procedures

Sepsis was elicited by a cecal ligation and puncture (CLP) technique described previously [14]. Briefly, rats were anesthetized by an intraperitoneal injection of pentobarbital (60 mg/kg body weight) and a midline abdominal incision was performed. The cecum was mobilized and ligated at its middle portion below the ileocecal valve, punctured once using a 21-gauge needle, and a small stool sample was squeezed out of the cecum to induce polymicrobial peritonitis. The abdominal wall was closed in two layers. Sham-operated mice underwent the same procedure, including opening of the peritoneum and exposing the bowel, but without ligation or needle perforation of the cecum. After surgery, the mice were resuscitated by a subcutaneous injection of pre-warmed (37 °C) normal saline (5 mL per 100 g body weight).

The experimental setup was adapted from our previous experiments [15]. Briefly, after the CLP or sham operation, a tracheotomy was performed. The left lateral tail vein and carotid artery were cannulated for an infusion of pentobarbital sodium (10 mg/kg/h) and heparin (2.5 U/mL/h), respectively, and the right lateral tail vein was cannulated for an infusion of cisatracurium or NaCl 0.9 %. Body fluid homeostasis was maintained by means of administration of an electrolyte solution (2.0 mL/kg/h). Body temperature was maintained at 37–37.5 °C (±0.5 °C) by using a heating lamp. Mean arterial pressure (MAP) was measured constantly. An electrode was implanted at the upper thigh level around the left sciatic nerve, which when stimulated, caused plantar flexion of the left foot. The plantar flexion twitch, induced by supramaximal stimulation, was measured before the infusion of cisatracurium or NaCl 0.9 %, after 1 h, and then every 3 h after. Cisatracurium infusions were titrated to achieve a 50 % reduction in twitch. Rats were ventilated using a volume-driven small-animal ventilator (V8S, Alcott Biotech, Shanghai, China) for 18 h with a tidal volume of 1 mL/100 g body weight and a respiratory rate of 80 breaths/min. Continuous care during MV included bladder expression, removal of airway mucus, and eye lubrication. Care was maintained throughout the experimental period at hourly intervals. Blood samples were obtained at 0, 3, 6, 12, and 18 h after CLP to determine levels of various inflammatory mediators. After 18 h, all animals were euthanized by an intraperitoneal injection of sodium pentobarbital (100 mg/kg). Costal diaphragm segments were removed to measure *in vitro* contractile properties. A section of the remaining costal diaphragm was frozen rapidly in liquid nitrogen and stored at −80 °C for Western immunoblot analysis.

Diaphragmatic Contractile Measurements

The diaphragm muscle was excised rapidly from the mid-costal region, and a diaphragm muscle strip (approximately 5 mm wide) with intact fibers inserted at the ribs and central tendon was used for isometric contractile measurements, as described previously [16]. Briefly, the diaphragm muscle strip was suspended vertically in a 37 °C

tissue bath containing Krebs solution: 137 mM NaCl, 4 mM KCl, 1 mM KH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, and 6.5 mM glucose. The solution was aerated continuously with a gas mixture of 95 % O_2/5 % CO_2, and a pH of 7.40 was maintained. The rib-end of the muscle was tied to a rigid supporter, and the central muscle tendon was connected to an isometric force transducer mounted on a micrometer. Two silver stimulating electrodes were placed parallel to the muscle strip. After equilibration for 15 min, isometric contractions were recorded at the optimal muscle length (L0) at which the maximal isometric tetanic force was observed. Stimuli were applied using a rectangular pulse duration of 0.2 ms and a train duration of 250 ms. To ensure supramaximal stimulation, the strips were stimulated at 20 % above voltage to obtain maximal forces. The signal was amplified and recorded using a data acquisition system (MPA 2000; Alcott Biotech, Shanghai, China). Once maximal stimulus intensity and the L0 for force production were determined, peak twitch tension, time to peak tension, and half-relaxation time were determined at L0 from a series of contractions induced by single-pulse stimuli. Maximal tetanic tension was produced by a supramaximal 250-ms stimulus train at 120 Hz. To measure the force-frequency response, each strip was stimulated with a 250-ms train at 10, 20, 40, 80, and 120 Hz, with at least a 2-min interval between each stimulus train. After the recovery period, the muscle was repositioned to its optimal length for tetanus recordings. The fatigability of each strip was assessed by 330-ms stimulations repeated at 25 Hz and applied every second for 5 min. The index of fatigability was calculated according to Burke [17] and was expressed as the percentage decrease in force developed by tetanus obtained at 120 s compared to the maximal initial tetanic force. Following the completion of all measurements, forces were normalized for muscle cross-sectional areas, which were obtained by dividing bundle weight by muscle specific density and optimal length.

Western Blot Analysis

The levels of HMGB1 in the diaphragm muscle were assayed by Western blot analysis. Briefly, protein extracts were resolved on 12 % SDS-polyacrylamide gels, then transferred onto poly(vinylidene fluoride) microporous membranes (Millipore, Bedford, MA, USA), blocked with 5 % skim milk, and probed with rabbit anti-HMGB1 polyclonal antibody (1:300; Abcam, San Diego, CA, USA) and anti-β-actin antibody (1:1000; Santa Cruz Technology, Santa Cruz, CA, USA) for 4–5 h. The blot was washed, exposed to horseradish peroxidase-linked secondary antibodies (1:1000; Golden Bridge, Beijing, China) for 1 h at room temperature, and finally detected by chemiluminescence (ECL, Amersham, Buckinghamshire, UK). The amount of protein in the blots was quantified using a densitometer and Multi Gauge version 3.0 software (FujiFilm Life Science, Tokyo , Japan).

Enzyme-Linked Immunosorbent Assay (ELISA)

The serum levels of TNF-α, IL-6, and HMGB1 were determined using enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions and guidelines.

Statistical Analysis

Values are expressed as the mean±standard deviation. The statistical analysis was performed with the SPSS version 17.0 statistical software package (SPSS Inc., Chicago, IL, USA). Data were tested for normality and equality of variance. Comparisons among the three groups for each dependent variable were performed using an analysis of variance (ANOVA) with a post hoc Newman-Keuls multiple comparison test. The level of statistical significance was set at $P<0.05$.

RESULTS

Systemic Responses to Treatment and Infusion Doses

The blood gas/pH homeostasis of the control group remained stable and was kept within the normal range throughout the experiment. In contrast, MAP and pH measured at dissection time in the CLP and Cis+CLP groups were significantly lower than those in the control group (Table 1). The results indicated that sepsis was induced successfully during the 18-h experimental protocol. The body weight of the rats was similar at baseline and after 18 h, and did not change significantly between the groups at the end of the experiments (data not shown). All the animals survived the entire experiment. The dose of sodium pentobarbital was similar in all groups. The mean cisatracurium dose was 3.38 mg/kg/h.

Diaphragm Contractile Properties

In vitro diaphragm peak twitch tension and maximal tetanic tension at its optimal length decreased significantly in the CLP group by 58 and 53 %, respectively, compared to values observed in the control rats. However, when Cis

Table 1. Blood Gas Data, Mean Arterial Pressure at Dissection Time, and Dose of Pentobarbital Sodium in each Group

	Control	CLP	Cis+CLP
Blood gasses			
PaO_2	137±28	128±27*	146±29*#
$PaCO_2$	38.2±3.3	47.6±2.7*	42.8±3.3*
pH	7.40±0.03	7.26±0.02*	7.30±0.03*
MAP	104.23±11.05	81.2±8.28*	85±13.19*
Dose of sedative			
Pentobarbital	16.03±0.61	16.54±0.44	15.95±0.81

Values are the mean±SD expressed in mmHg for blood gases and in mg/kg/h for the doses of pentobarbital sodium
MAP mean arterial pressure
*$P<0.05$ compared to the control group; # $P<0.05$ compared to the CLP group

was combined with CLP, the peak twitch tension and maximal tetanic tension improved significantly by 27 and 23 %, respectively, compared to the values observed with CLP alone. No significant difference was observed in the time to peak tension or half-relaxation time in all groups. After 18 h, the fatigability index was significantly lower in the CLP group (29 % of the initial force) compared to the control or Cis+CLP groups (46 and 34 % of the initial force, respectively), evidencing greater fatigability; administration of Cis attenuated the fatigability (Table 2).

Diaphragm force-frequency curves are shown in Fig. 1. Compared to the control group, the CLP group demonstrated a downward shift in the force-frequency curve, which suggested significantly reduced force generation. The administration of cisatracurium over 18 h attenuated the loss of force production.

HMGB1 Expression in the Rat Diaphragm

The expression of diaphragm HMGB1 was significantly increased in the CLP group compared to the control group (Fig. 2). Compared to the CLP group, HMGB1 protein expression was markedly downregulated in the Cis+CLP group ($P<0.05$).

Table 2. *In Vitro* Isometric Diaphragmatic Contractile Properties

	Control	CLP	Cis+CLP
Pt (mN/cm^2)	6.32±0.75	2.67±0.37*	3.39±0.42*#
P0 (mN/cm^2)	13.37±1.46	6.23±0.87*	7.67±1.01*#
CT (ms)	63.52±5.46	65.73±5.42	64.36±7.15
$RT_{1/2}$ (ms)	40.78±6.06	42.87±7.43	41.22±4.64
Fatigability Index	0.49±0.05	0.29±0.06*	0.36±0.06*#

Pt peak twitch force, *P0* maximum tetanic tension, *CT* contraction time to peak force, $RT_{1/2}$ half relaxation time.
*$P<0.05$ compared to the control group; # $P<0.05$ compared to the CLP group

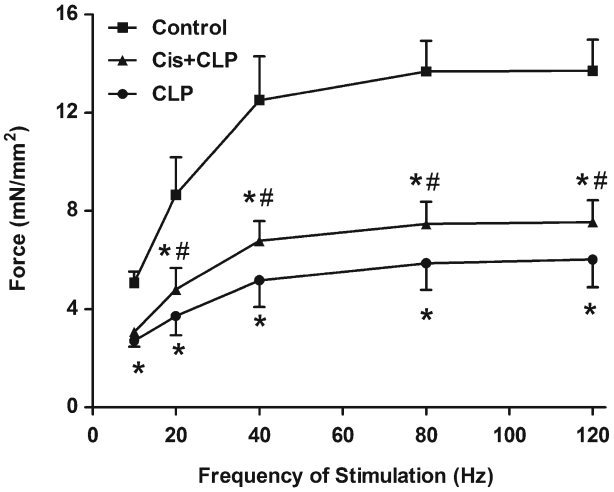

Fig. 1. Force-frequency curves from the diaphragm strips. Force-frequency curves demonstrate that CLP reduces the diaphragm-specific force production at different stimulation frequencies compared to a sham operation. The loss of force generation induced by CLP was partially attenuated by the administration of cisatracurium. *$P<0.05$ compared to the control group; #$P<0.05$ compared to the CLP group.

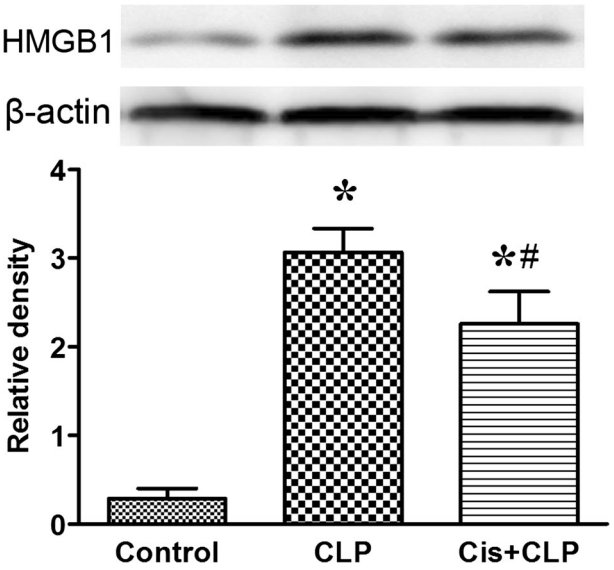

Fig. 2. Changes in the expression of high-mobility group box 1 (HMGB1) in rat diaphragm. A comparison of the relative densities of HMGB1 and β-actin are shown for the control, CLP, and Cis+CLP groups. Data are shown as the mean±SD. *$P<0.05$ compared to the control group; #$P<0.05$ compared to the CLP group.

Serum Levels of Various Inflammatory Mediators

We investigated the effect of Cis on CLP-induced expression of the proinflammatory factors IL-6, TNF-α, and HMGB1. After the CLP procedure, serum was sampled at various time points over the course of 18 h. The IL-6 levels were elevated after the CLP procedure in both the CLP and Cis+CLP groups, but the levels were significantly lower in the Cis+CLP group (Fig. 3a). Likewise, TNF-α was induced by CLP in both groups, but Cis attenuated the induction (Fig. 3b). Serum levels of HMGB1 increased steadily over time after the CLP procedure in the CLP and Cis+CLP groups; again, Cis significantly reduced the levels of HMGB1 (Fig. 3c). Serum IL-6, TNF-α, and HMGB1 levels did not increase in the control group.

DISCUSSION

As the main respiratory muscle, the diaphragm plays a very important role in the process of breathing. Diaphragm dysfunction induced by sepsis often results in difficulty weaning critically ill patients from mechanical ventilation and even high mortality. Studies confirmed that sepsis induced more abundant cytokines in the diaphragm compared to the limb muscles, which indicates that respiratory muscles are more susceptible to sepsis-induced injury [18]. Therefore, observing diaphragmatic function in a setting of sepsis is effective for exploring muscle weakness. In agreement with previous studies [19–21], we have demonstrated that sepsis caused significant diaphragmatic dysfunction in a rat model.

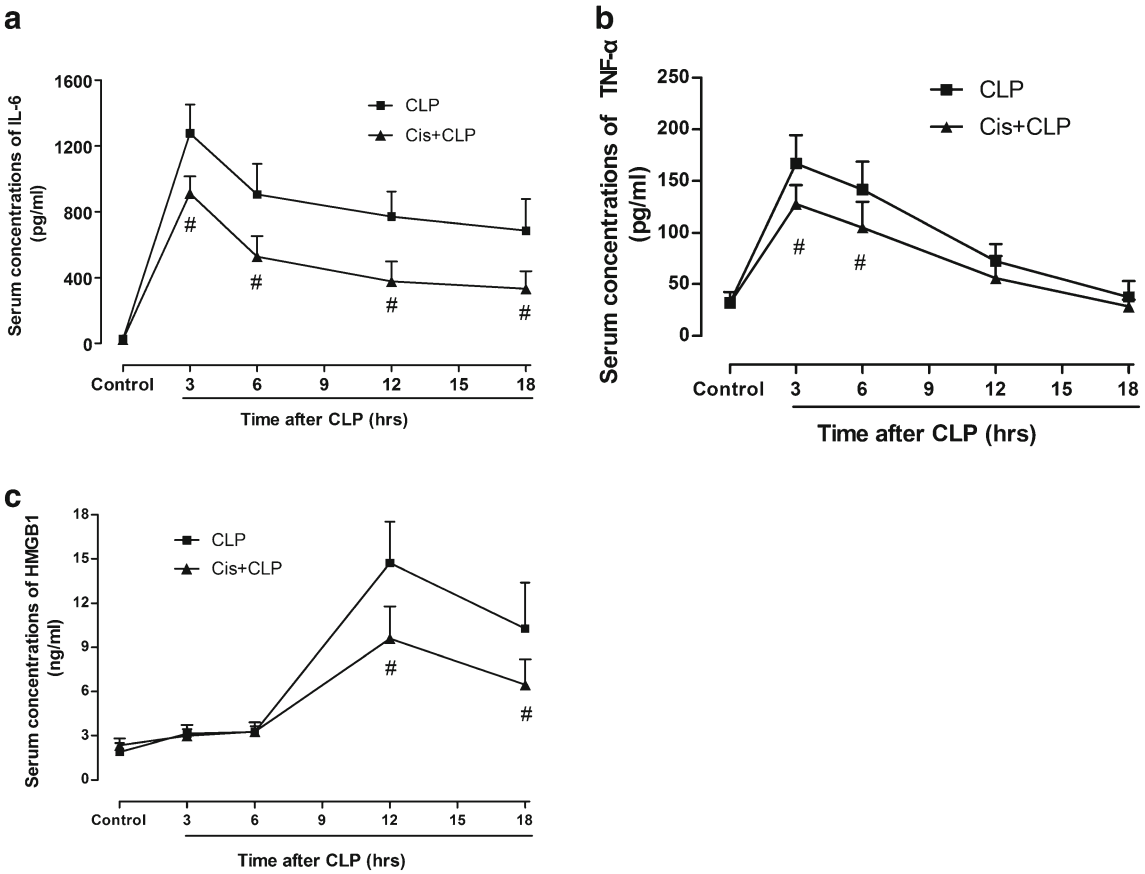

Fig. 3. Increased interleukin-6, tumor necrosis factor-α, and high-mobility group box 1 serum levels after cecal ligation and puncture in rats. Serum levels of **a** interleukin-6 (IL-6), **b** tumor necrosis factor-α (TNF-α), and **c** high-mobility group box 1 (HMGB1) were determined in untreated rats after cecal ligation and puncture (CLP group) and in rats treated with Cis after cecal ligation and puncture (Cis+CLP group). Data are presented as the mean±SD; $^{\#}P<0.05$ compared to the CLP group.

Various animal models have been developed to assess diaphragm function in sepsis; the most frequently used being the cecal ligation and puncture (CLP) model in rodents. In this model, sepsis originates from a polymicrobial infectious focus within the abdominal cavity, followed by bacterial translocation into the blood compartment, which then triggers a systemic inflammatory response [22]. We used this model to mimic pathophysiological changes typically seen in septic patients and observed that the concentrations of TNF-α and IL-6 peaked at 3 h after CLP, while serum HMGB1 reached its peak at 12 h after CLP. The excessive production of these cytokines can initiate secondary responses in organs and propagate tissue damage and dysfunction [23, 24]. We also found elevated levels of HMGB1 protein in the diaphragm 18 h after CLP (the conditions of late sepsis), which is in line with the notion that diaphragm muscle cells are able to produce cytokines in response to sepsis [18, 25].

The main finding of the current study is that the early administration of cisatracurium during an 18-h period post-CLP attenuated sepsis-induced diaphragmatic dysfunction, as evidenced by increased diaphragm maximal twitch and tetanic force production compared to CLP rats. The mechanisms underlying the protective effect of cisatracurium on sepsis-induced diaphragmatic dysfunction remain speculative. In our understanding, it may be explained as follows. First, the early administration of cisatracurium could decrease the proinflammatory response. In the present study, we found decreased expression of TNF-α, IL-6, and HMGB1 in serum, as well as an alleviation of HMGB1 protein expression in the diaphragm. The results are in agreement with those of a previous study, which demonstrated that the early use of NMBDs decreased the proinflammatory response associated with acute respiratory distress syndrome and MV [11]. Second, the infusion of NMBDs may improve chest wall compliance, prevent respiratory dyssynchrony, reduce peak airway pressures, and improve oxygenation, thereby limiting the risk of lung and diaphragm injury. A previous study reported that facilitated MV in septic animals prevented sarcolemmal damage to the diaphragm and protected against diaphragm injury [26]. Thus, satisfactory muscle relaxation may mitigate sepsis-induced weakness. Taken together, the protective effect of cisatracurium on sepsis-induced diaphragm dysfunction may be related to the inhibition of cytokine responses and the facilitation of MV, although further studies are needed to confirm this.

Our findings suggest that cisatracurium is an effective drug for the prevention of sepsis-induced diaphragmatic dysfunction. The effective dose of cisatracurium used in this study is comparable with that used by clinicians in the ICU [27]. However, it should be noted that our study may have several possible limitations. First, various NMBDs are used in ICU settings, but we only investigated cisatracurium in this study. Therefore, whether other NMBDs, including rocuronium and atracurium, exert different effects on diaphragm function needs to be further investigated. Second, further studies should be conducted to investigate the cisatracurium dose-response relationship with protective effect in this model, which will provide more convincing evidence of a cause-effect relationship.

In conclusion, the present results indicate that the early infusion of cisatracurium attenuates sepsis-induced diaphragm dysfunction, and this may be attributable to its anti-inflammatory action. This finding is worthy of further clinical study in situations where cisatracurium is an ideal option for maintaining muscle relaxation in septic patients at risk of myopathy.

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Conflicts of Interest. None

Author's Contribution. Jihong Jiang and Bin Yang helped conduct the study, analyze the data, and write the manuscript. Guangwei Han helped write the manuscript; Meirong Yang helped analyze the data; and Shitong Li helped design the study and write the manuscript.

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