

Original Article

Combined administration of a sedative dose sevoflurane and 60% oxygen reduces inflammatory responses to sepsis in animals and in human PMBCs

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Abstract: Our study aims to investigate the effects of the inhalation of subanesthetic doses of sevoflurane combined with oxygen on sepsis. Male Sprague-Dawley rats or Male ICR/Km mice underwent caecal ligation and puncture (CLP) or intraperitoneal injection of lipopolysaccharide (LPS) to induce sepsis, while sham rats were used as control. Then, rats were treated with the inhalation of sevoflurane in oxygen; and air or 100% oxygen was used as control. Seven-day survival, lung injury and inflammatory factors were assessed. In this *in vitro* experiment, we obtained RAW264.7 macrophages and human peripheral blood mononuclear cells (PBMCs) incubated by LPS or plasma from septic patients to explore the NF- κ B pathway in the effect of the inhalation of sevoflurane combined with oxygen in sepsis. In this study, we found that the inhalation of 0.5 MAC of sevoflurane in 60% oxygen was the best protocol for protecting against lethality resulting from sepsis and ALI, and there was a time window for these protective effects. We also founded that 0.5 MAC of sevoflurane in 60% oxygen inhibited the nuclear translocation of NF- κ B in human PBMCs induced by LPS or plasma from septic patients. The subanesthesia dose sevoflurane in 60% oxygen may reduce sepsis-induced inflammatory responses in animals and in PBMCs, and the inhibition to the activation of the NF- κ B pathway may contribute to this protection.

Keywords: Acute lung injury, sepsis, sevoflurane, NF- κ B, human peripheral blood mononuclear cells

Introduction

Sepsis is one of the most elusive syndromes in medicine. Patients with sepsis constitute a large proportion of the critically ill population. Although outcomes have improved [1], mortality remains higher than 25-30%, and may even reach 40-50% when shock is present [2]. Over 40% of individuals with sepsis develop acute lung injury (ALI), which markedly worsens patient prognosis, and increases intensive care unit mortality from 11% to 38% in patients with septic shock [3].

Bacterial pathogens initiate systemic inflammation by activating cytokine networks and inducing the expression of pro-inflammatory genes. This is a process that is principally mediated by activating an inducible transcription factor

such as NF- κ B. Previous studies have suggested that activated NF- κ B is a driving force in the initiation and progression of systemic inflammation and septic pathophysiology [4, 5]. Hence, the regulation of inflammatory response through the NF- κ B signaling pathway has been a focus of studies.

Our earlier study indicated that the inhalation of 100% oxygen provides protection against zymosan-induced sterile sepsis in mice [6], which was confirmed by various animal models of sepsis [7, 8]. In addition, the anesthetic dose (3.3%-4%) of sevoflurane and hexafluoroisopropanol, a water-soluble primary sevoflurane metabolite, has also been reported to markedly reduce CLP-induced seven-day mortality in a model of murine septic peritonitis; which is accompanied by the reduction of end-organ damage [9, 10].

However, the application of hyperoxia or the anesthetic dose of sevoflurane to critically ill patients is limited, because long-term hyperoxia treatment can induce oxygen toxicity [11] and the anesthetic dose of sevoflurane may increase mortality in sepsis [12]. A number of trials have shown the effectiveness of using lower doses of volatile anesthetic agents for ICU sedation, with shorter wake up and extubation time, lesser duration of mechanical ventilation, and faster discharge from hospitals [13, 14]. Furthermore, a combination of volatile anesthetics with different concentrations of oxygen, including 100% oxygen, has often been observed during clinical anesthesia. Therefore, we hypothesize that the combined administration of a subanesthetic dose of sevoflurane and different concentrations of oxygen may have protective effects on sepsis and its relevant ALI.

In the present study, we explored the optimal-ratio hybrid gas of sevoflurane and oxygen for protecting against experimental sepsis. We suggest that this protective mechanism was the inhibition of inflammation via the NF- κ B signaling pathway, during *in vitro* sepsis induced by lipopolysaccharide (LPS) or plasma from septic patients. Our study provides a new therapeutic approach to improve sepsis and its relevant ALI, especially for the clinical treatment of sepsis.

Materials and methods

Animals and animal models of sepsis

The Institutional Animal Care and Use Committee of the Fourth Military Medical University approved all animal protocols. All experiments were performed according to the National Institutes of Health guidelines. Male ICR/Km (Institute of Cancer Research, NIH, USA/Kunming Institute of Zoology, China) mice (20-25 g) and Sprague-Dawley (SD) rats (250-300 g) were obtained from the Experimental Animal Center of the Fourth Military Medical University. Polymicrobial sepsis was induced in anesthetized rats by cecal ligation and puncture (CLP), according to literature [15]. Briefly, a longitudinal skin midline incision was performed after disinfecting the shaved lower abdomen and exposing the cecum. The cecum was ligated at half of the distance between the distal pole and the base of the cecum, and was perforated ('through-and-through') with a 21-

gauge needle. Then, a small amount of feces was expressed through the puncture wound. Sham animals underwent the same procedure without CLP. At last, the abdominal incision was closed in two layers. After surgery, the animals were resuscitated in a warm cage.

An endotoxin sepsis was performed on mice by intraperitoneal injection of LPS (Sigma Chemical, St. Louis, MO, USA; 30 mg/kg). The same volume of NS was given to animals in the control group through the same route.

Humans

Between July 2014 and September 2014, blood were sampled from patients with or without sepsis, who were admitted to *Xijing Hospital, Fourth Military Medical University* (Xi'an, China), as part of a study on the effects of oxygen and oxygen plus volatile anesthetics on experimental sepsis (ClinicalTrials.gov NCT02185118). A written informed consent was obtained from the proxy decision makers of patients before the study inclusion. After these individuals regained their decision-making capacity, they were informed of their participation, and were re-consented according to institutional policies. Exclusion criteria were established according to the diagnostic criteria for sepsis based on literature [16]. All human protocols were approved by the Institutional Review Board of Xijing Hospital, Fourth Military Medical University.

Treatment of oxygen or sevoflurane in oxygen

Animals were placed in a sealed plexi glass chamber with an inflow and outflow hose. Oxygen and sevoflurane (Baxter Healthcare, Puerto Rico) were delivered to the chamber through a tube, and carbon dioxide was removed from the chamber gases with baralyme. A gas analyzer (Brüel and Kjaer, Naerum, Denmark) was used to continuously monitor the concentration of oxygen and sevoflurane in the outflow hose of the chamber, which was maintained at a predetermined level during treatment.

Wet-to-dry weight ratio in lungs (lung W/D ratio)

At predetermined time points, the harvested left wet lung was immediately weighed. Then, it was placed in an oven for 24 hours at 80°C, and was re-weighed when it was dried. The lung weight wet-to-dry ratio was recorded.

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Protein production in bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was obtained by cannulating the trachea of rats with a 16-gauge catheter and lavaging with phosphate buffer saline (pH 7.4, with a total of 5 ml). Lavage samples were centrifuged (1,000 g, 10 minutes, at 4°C), and the supernatant was stored at -80°C. Total protein was measured using the BCA Protein Assay Kit according to manufacturer's instructions. Albumin level was measured by enzyme-linked immunosorbent assay (ELISA), as previously described [17].

Cytokines

The cytokines in serum or the intraperitoneal lavage fluid were detected using specific ELISA kits (R&D Systems Inc., Minneapolis, Minnesota, USA), according to manufacturer's instructions. All standards and samples were run in triplicate.

Lung myeloperoxidase (MPO) activity

MPO activity, an indicator of neutrophil infiltration in lung tissues, was detected in homogenized lung supernatants, as previously reported [18] using an MPO Assay Kit (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China), according to manufacturer's instructions.

Cell culture

RAW264.7 cell lines were obtained from the Department of Microbiology, School of Basic Medicine, Fourth Military Medical University. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, USA) supplemented with 10% (vol/vol) heat-inactivated fetal calf serum and 1% penicillin and streptomycin. The incubator was set at 37°C with a humidified atmosphere containing 5% CO₂.

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood under endotoxin-free conditions through Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ, USA) density centrifugation, according to manufacturer's protocol. The isolated cells were suspended in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, New York, USA) and 1% penicillin/streptomycin

(Beyotime Biotechnology, Shanghai, China); and subsequently, these cells were cultured in 24-well plates at 37°C for six hours. Then, these cultures were washed to remove non-adherent cells. The purity of CD14-positive monocytes was always more than 90%, as assessed by immunofluorescence staining and counting [19].

Western blotting

The protein from RAW264.7 cell samples was directly extracted based on manufacturer's standard protocols (Beyotime Biotechnology, Shanghai, China). Then, cytoplasmic and nuclear protein fractions were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, Shanghai, China), according to the manufacturer's protocol for western blot analysis. Primary rabbit antibodies for nuclear factor-kappa B p65 subunit (p65), phosphorylated NF-κB p65 (p-p65), phosphorylated inhibitor of kappa B alpha (p-IκBα), and phosphorylated IκB kinase (IKK)-α/β (p-IKKα/β) (Cell Signalling Technology, Boston, USA) proteins were used to detect p65, p-p65, p-IκBα and p-IKKα/β protein expression. Then, the primary rabbit antibodies for proliferating cell nuclear antigen (PCNA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), IKKα, IKKβ, and IκBα proteins (Cell Signaling Technology, Boston, USA) were used to detect PCNA, GAPDH, IKKα, IKKβ, and IκBα proteins, which were used as control.

Immunofluorescence analysis

For immunofluorescence assays, RAW264.7 cells or freshly isolated PBMCs were seeded on glass coverslips in 24-well plates and cultured. Immunofluorescence staining was performed using the NF-κB p65 nuclear translocation kit (Beyotime, Shanghai, China), according to manufacturer's instructions. Briefly, cells were fixed with 4% paraformaldehyde for 15 minutes, and the slides were washed three times with PBS. After processing with blocking solution for one hour at room temperature, cells were incubated with anti-NF-κB p65 antibody overnight at 4°C. The slides were washed three times with PBS and incubated with fluorescein-conjugated secondary antibody for one hour at room temperature. DAPI staining was used for the counterstaining of the nucleus. Fluorescent images of the coverslips were obtained by confocal microscopy (Olympus, Tokyo, Japan).

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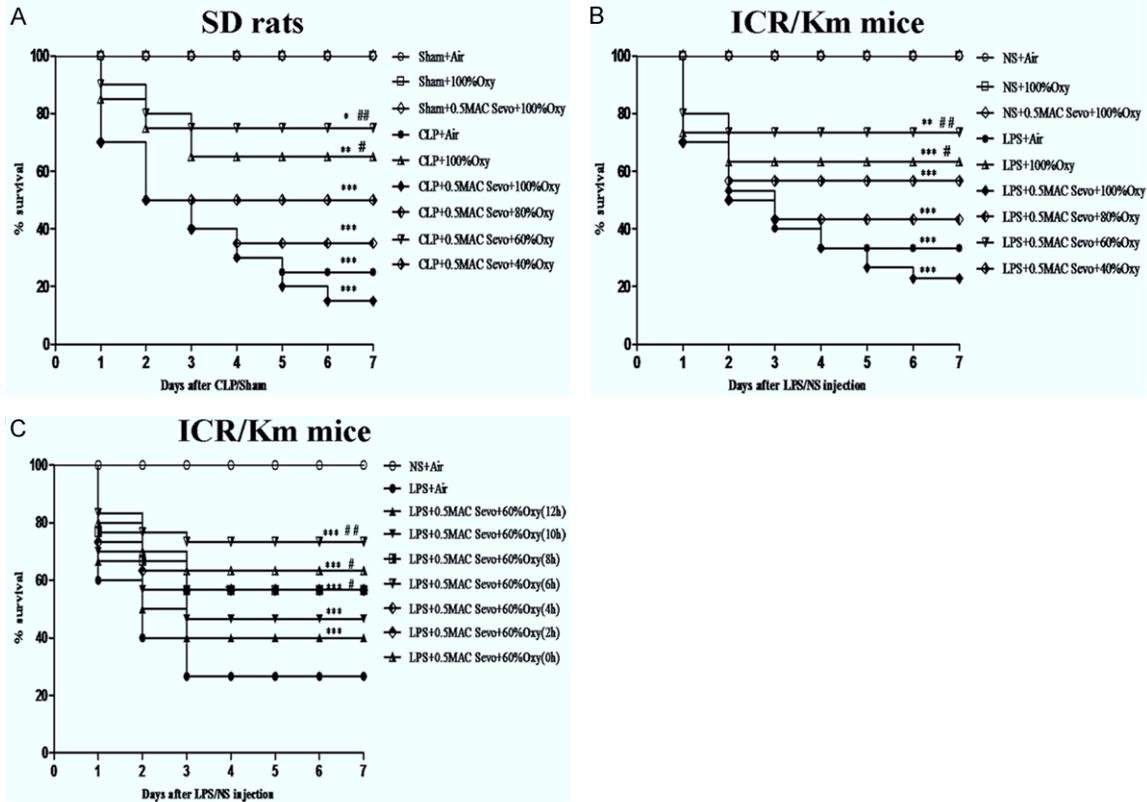


Figure 1. Inhalation of 0.5 MAC sevoflurane in 60% oxygen improved sepsis-induced lethality. Sepsis was induced by CLP (A) or by intraperitoneal injection of LPS (B, C). (A) Dose-dependent effects on CLP-induced sepsis in SD rats. Treatment was performed on animals by inhalation of 100% oxygen or 0.5 MAC sevoflurane in 40%, 60%, 80% and 100% oxygen for 2 h at 6 h after the challenge, respectively. The survival rate was observed for 7 days. The values are expressed as survival percentage ($n = 20$ in each group). (B) Dose-dependent effects on LPS-induced sepsis in ICR/Km mice. Treatment was performed on animals by inhalation of 100% oxygen or 0.5 MAC sevoflurane in 40%, 60%, 80% and 100% oxygen for 2 h at 6 h after the challenge, respectively. The survival rate was observed for 7 days. The values are expressed as survival percentage ($n = 30$ in each group). (C) Time-dependent effects on LPS-induced sepsis in ICR/Km mice: Treatment was performed on animals by inhalation of 0.5 MAC sevoflurane in 60% oxygen for 2 h at 0, 2, 4, 6, 8, 10 and 12 h after the challenge, respectively. The survival rate was observed for 7 days. The values are expressed as survival percentage ($n = 30$ for each group). Data are expressed as mean \pm s.e.m. ($n = 6$ for each group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. NS/Sham+Air group; # $P < 0.05$, ## $P < 0.01$ vs. LPS/CLP+Air group, respectively. CLP: cecal ligation and puncture; CFUs: colony forming units; h: hour; LPS: lipopolysaccharide; MAC: minimum alveolar concentration; NS: normal saline; Oxy: oxygen; Sevo: sevoflurane.

Statistical analysis

The measurement data and histological score are expressed as mean \pm standard error of the mean (SEM). Intergroup differences in the levels of biochemical parameters, inflammatory cytokines, and the number of neutrophils were tested by one-way ANOVA, followed by the least significant difference test for multiple comparisons. Intergroup differences of pathologic scores were tested using the Kruskal-Wallis H method, followed by the Nemenyi test for multiple comparisons. Survival studies were analyzed using the log-rank test. Survival rates were expressed in percentage. The statistical analysis was performed using the SPSS 20.0

software (SPSS Inc, Chicago, USA). In all tests, $P < 0.05$ was considered statistically significant.

Results

The inhalation of 0.5 MAC of sevoflurane in 60% oxygen protected against sepsis-induced lethality

CLP, which is an animal model of bacterial peritonitis, has been regarded as the “gold standard” animal model of sepsis [20]. In CLP-challenged rats, a significant improvement of the 7-day survival rate was observed only in rats treated with 0.5 MAC of sevoflurane in 60% oxygen ($P < 0.01$) (Figure 1A); which was also

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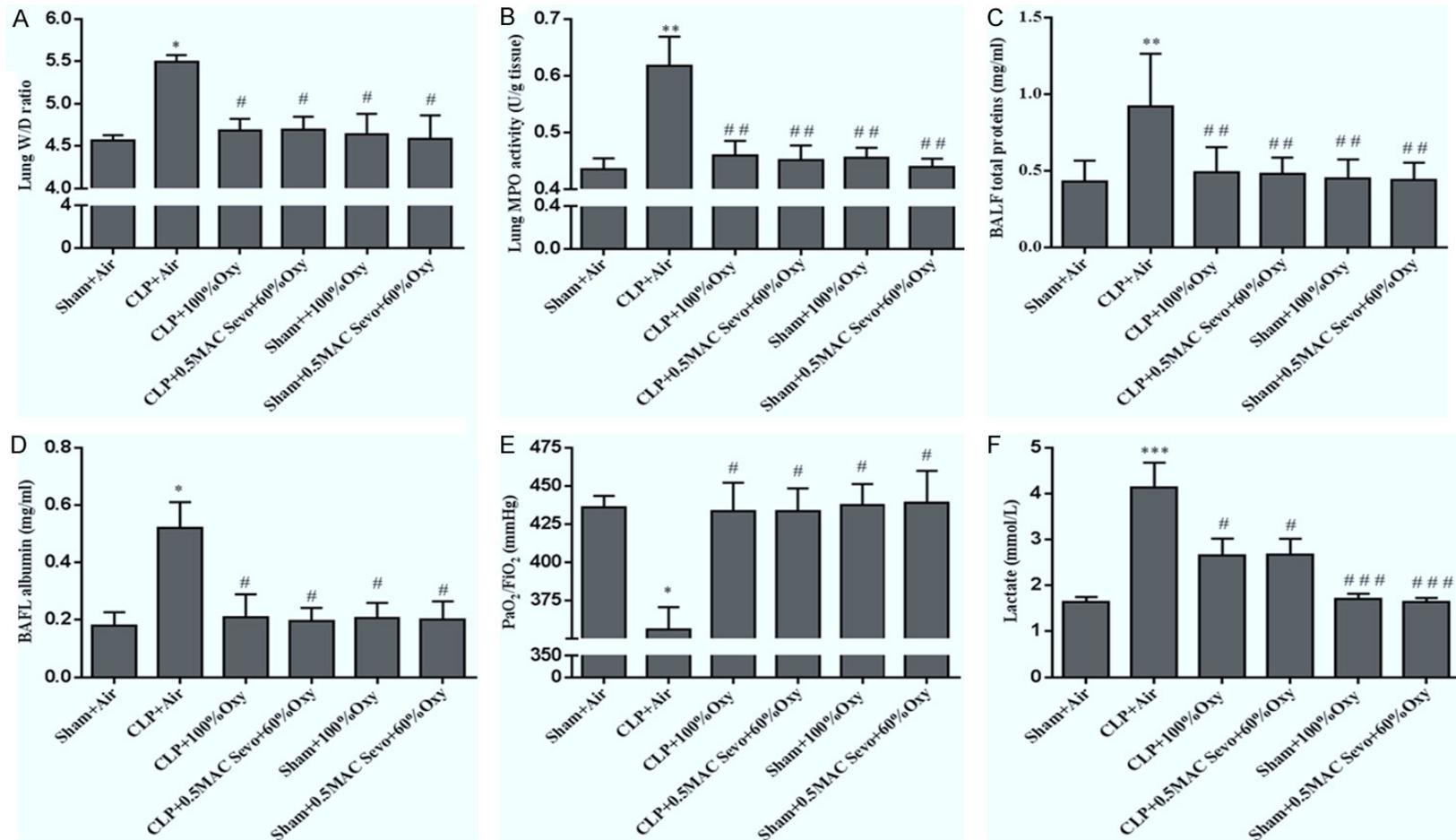


Figure 2. The 0.5 MAC sevoflurane in 60% oxygen mitigated lung injury resulting from CLP-induced sepsis in rats. Assessment of lung injury was done by measuring production of total protein and albumin in BALF, lung wet to dry ratio and MPO activity at 24 h after CLP/Sham-operation. A: Lung wet to dry ratio. B: Lung MPO activity. C: BALF total protein production. D: BALF albumin production. E: Oxygenation indices (PaO₂/FiO₂). F: Lactate in arterial blood. Data are expressed as mean ± s.e.m. (n = 6 for each group). *P < 0.05, **P < 0.01, ***P < 0.001 vs. Sham+Air group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. CLP+Air group. BALF: bronchoalveolar lavage fluid; CLP: cecal ligation and puncture; h: hour; MAC: minimum alveolar concentration; MPO: myeloperoxidase; Oxy: oxygen; Sevo: sevoflurane.

observed in the murine model of sepsis induced by LPS ($P < 0.05$ or 0.01) (**Figure 1B**). LPS is the major component of the outer membrane of Gram-negative bacteria, which are considered as one of the predominant organisms that causes sepsis [21]. It was also observed that treatment with 0.5 MAC of sevoflurane in 60% oxygen for two hours significantly increased the 7-day survival rates of animals with LPS-induced sepsis only when it was performed at 0, 2, 4, 6 and 8 hours after LPS injection ($P < 0.05$ or 0.01); and the inhalation of 0.5 MAC of sevoflurane in 60% oxygen for two hours after six hours LPS injection was the best therapy for sepsis (**Figure 1C**). The above results suggest that 0.5 MAC of sevoflurane in 60% oxygen protects against lethality resulting from sepsis induced by CLP and LPS, and there was a time window on the protective effects induced by 0.5 MAC of sevoflurane in 60% oxygen.

Inhalation of 0.5 MAC of sevoflurane in 60% oxygen protected against sepsis-induced ALI

ALI, which is typically observed in individuals with sepsis, is an inflammatory disease. Dysregulated inflammatory response in lungs, as well as the altered permeability of alveolar endothelial and epithelial barriers, remain as central pathophysiologic concepts in ALI and acute respiratory distress syndrome (ARDS) [22]. In this study, we evaluated the effects of 0.5 MAC of sevoflurane in 60% oxygen on lung injury resulting from CLP-induced sepsis by determining the parameters of lung injury. In CLP-challenged rats, the treatment of 0.5 MAC of sevoflurane in 60% oxygen significantly reduced lung W/D ratio ($P < 0.05$, **Figure 2A**), attenuated lung MPO activity ($P < 0.01$, **Figure 2B**), and reduced the production of total protein ($P < 0.01$, **Figure 2C**) and albumin in BALF ($P < 0.05$, **Figure 2D**). Furthermore, it was also observed that the inhalation 0.5 MAC of sevoflurane in 60% oxygen significantly increased oxygenation indices ($P < 0.05$, **Figure 2E**) and decreased the level of lactate in arterial blood ($P < 0.05$, **Figure 2F**). Additionally, the treatment of 0.5 MAC of sevoflurane in 60% oxygen significantly improved arterial pH value, arterial partial pressure of oxygen (PaO_2) and arterial partial pressure of carbon dioxide (PaCO_2) ($P < 0.05$ or $P < 0.01$, **Supplementary Figure 1A-C**). It was also found that the treatment of 0.5 MAC of sevoflurane in 60% oxygen alleviated CLP-induced morphological changes in lungs, and significantly inhibited an increase in pathological score for lung injury resulting from CLP ($P <$

0.01), moreover, neutrophil accumulation was reduced and the number of neutrophils significantly decreased in rat lungs induced by CLP ($P < 0.001$), which these results are presented in **Supplementary Figure 2**.

The above results demonstrate that treatments with 0.5 MAC of sevoflurane in 60% oxygen attenuate lung injury resulting from CLP-induced sepsis by inhibiting lung inflammation, and protecting alveolar endothelial and epithelial barriers.

Inhalation of 0.5 MAC of sevoflurane in 60% oxygen protected against CLP-induced sepsis through the regulation of inflammatory responses

Severe sepsis is characterized by the dysregulation of immune inflammatory response and disorder of coagulation function [23]. It has been believed that the imbalance of immune inflammatory reaction is a leading cause of death in septic patients [24]. In order to study whether this immune inflammatory mechanism contributed to these protective actions, the effects of 0.5 MAC of sevoflurane in 60% oxygen on CLP-induced changes in inflammatory factors in serum or peritoneal lavage fluid were examined at 24 hours after CLP in rats.

In CLP-challenged rats, both treatments of 0.5 MAC of sevoflurane in 60% oxygen significantly inhibited the increase in serum levels of proinflammatory factors IL-1 β , IL-6, TNF- α and HMGB1 at 24 hours after CLP ($P < 0.05$ or < 0.01 ; **Figure 3A-D**), and promoted an increase in the level of anti-inflammatory factor IL-10 in serum at 24 hours after CLP ($P < 0.05$, **Figure 3E**). Similar results were observed in peritoneal fluid (**Supplementary Figure 3**). The above results suggest that both treatments of 0.5 MAC of sevoflurane in 60% oxygen protect animals against CLP-induced sepsis through the inhibition of peritonitis and systemic inflammation after CLP.

The treatment of 0.5 MAC of sevoflurane in 60% oxygen inhibited the activation of the NF- κ B pathway in RAW264.7 cells after LPS stimulation

NF- κ B is a key regulator of inducible gene expression in the immune system, and is crucial to the signaling networks involved in sepsis. Previous studies have suggested that increasing NF- κ B activity is associated with the development and mortality of sepsis [5, 25].

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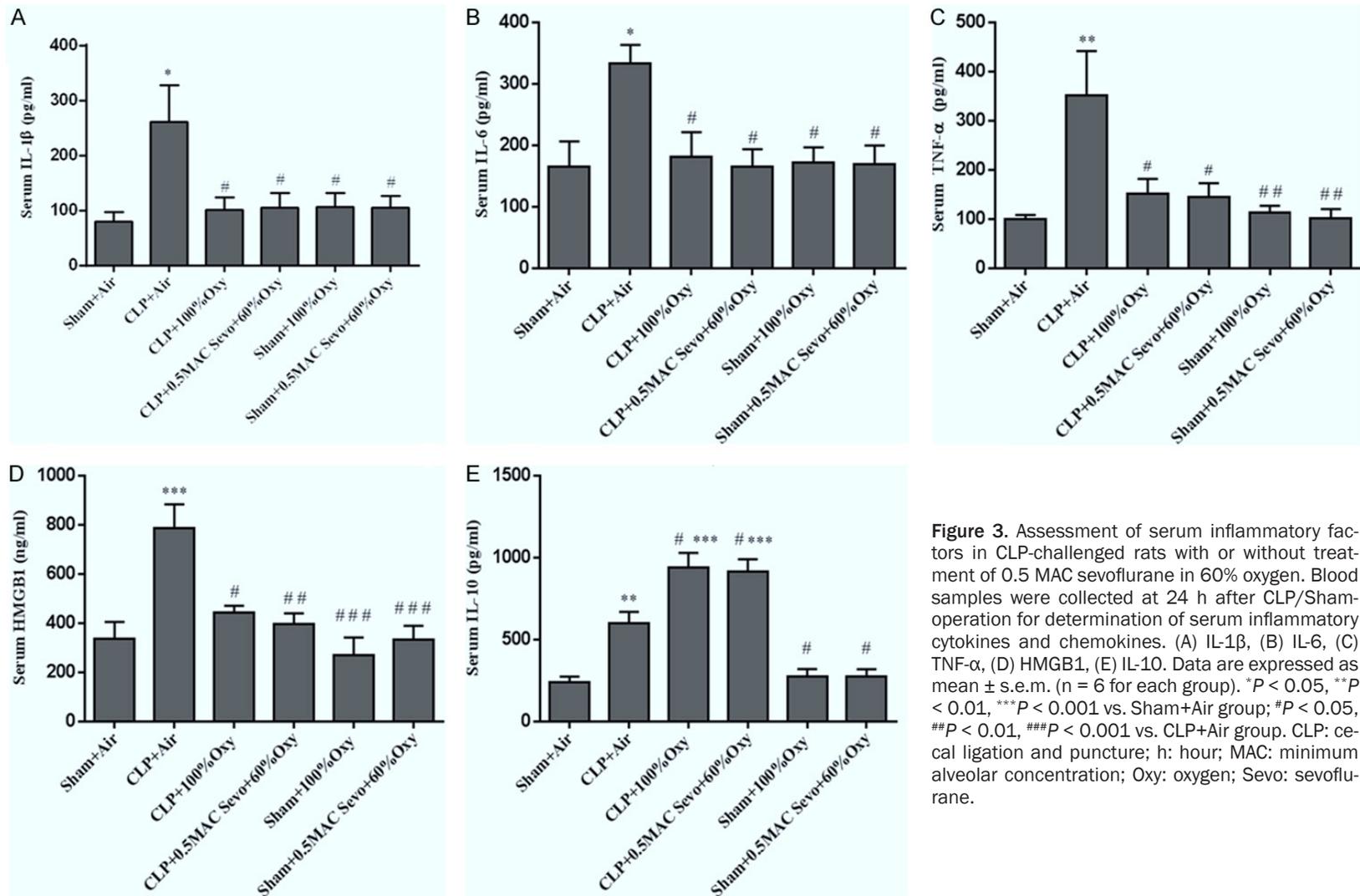
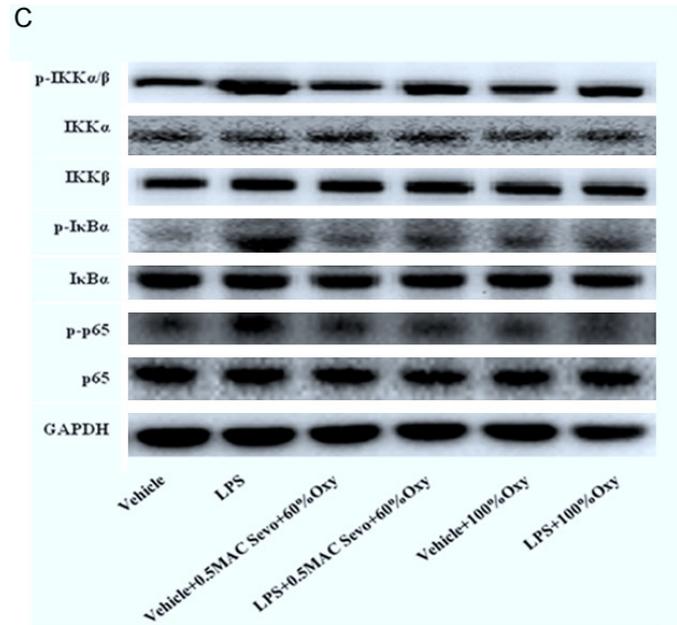
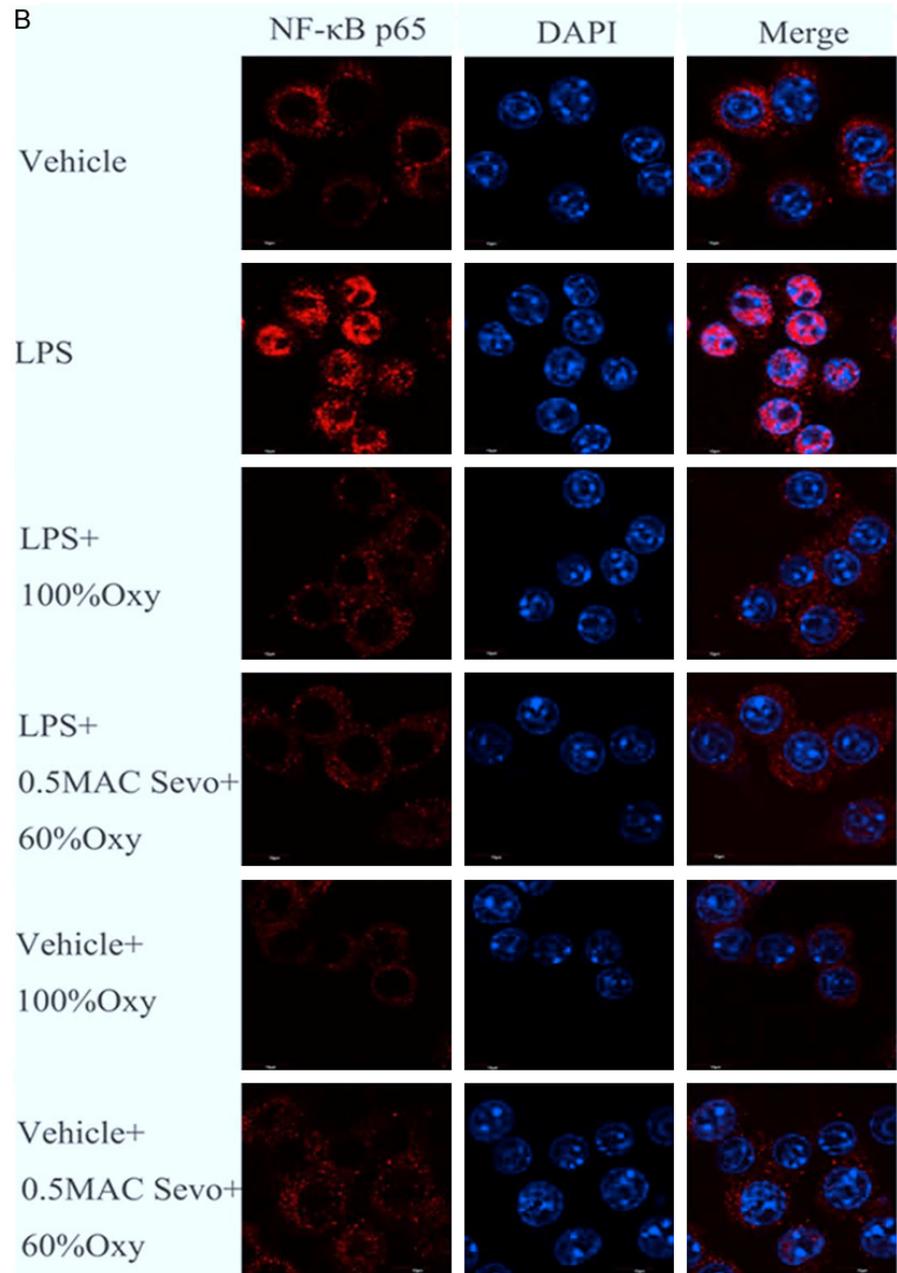
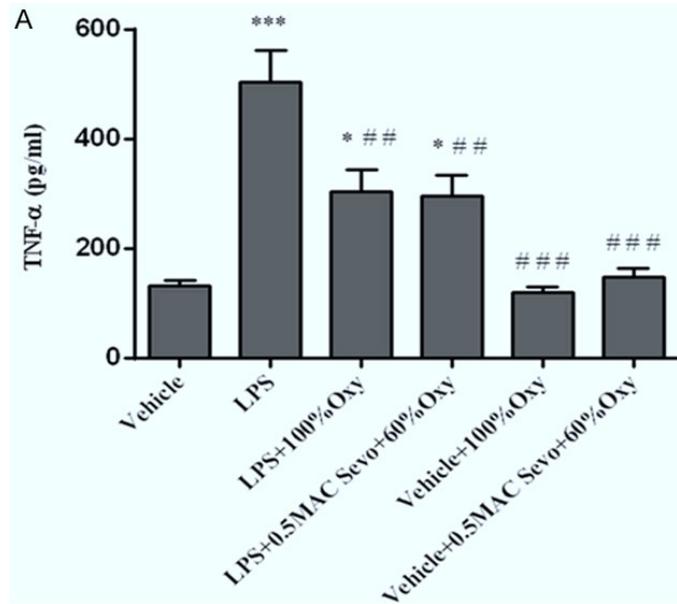


Figure 3. Assessment of serum inflammatory factors in CLP-challenged rats with or without treatment of 0.5 MAC sevoflurane in 60% oxygen. Blood samples were collected at 24 h after CLP/Sham-operation for determination of serum inflammatory cytokines and chemokines. (A) IL-1 β , (B) IL-6, (C) TNF- α , (D) HMGB1, (E) IL-10. Data are expressed as mean \pm s.e.m. (n = 6 for each group). * P < 0.05, ** P < 0.01, *** P < 0.001 vs. Sham+Air group; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. CLP+Air group. CLP: ce-cal ligation and puncture; h: hour; MAC: minimum alveolar concentration; Oxy: oxygen; Sevo: sevoflurane.

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Figure 4. The 0.5 MAC sevoflurane in 60% oxygen protected RAW264.7 cells against LPS-induced in-vitro sepsis through inhibiting activation of NF- κ B pathway. Induction of an in-vitro sepsis was performed on RAW264.7 macrophage cell line via incubation with 100 ng/ml LPS for at least 2 h. Cells were treated with 100% oxygen or 0.5 MAC sevoflurane in 60% oxygen for 2 h beginning 0.5 h after LPS stimulation. A: Treatment of 100% oxygen or 0.5 MAC sevoflurane in 60% oxygen significantly inhibited an increase of TNF- α concentration in cell culture supernatant by LPS stimulation. Data are expressed as mean \pm s.e.m. (n = 6 for each group). * P < 0.05, ** P < 0.01, *** P < 0.001 vs. vehicle group; ## P < 0.01, ### P < 0.001 vs. LPS group. B: Fluorescence images of NF- κ B p65 subunit nuclear translocation captured on a confocal laser scanning microscope (\times 240). C: Expressions of cellular p-IKK α/β , p-IkB α , p-p65, as well as IKK α/β , IkB α , p65 and GAPDH proteins were determined by western blot. And the expressions of IKK α/β , IkB α , p65 and GAPDH in cell were used as the controls. Each represents three independent experiments. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; h: hour; LPS: lipopolysaccharide; MAC: minimum alveolar concentration; NF- κ B: nuclear factor kappa B; Oxy: oxygen; p-IkB α : phosphorylated inhibitor of nuclear factor kappa B α ; p-IKK α/β : phosphorylated IkB kinase α/β ; p-p65: phosphorylated p65 subunit of nuclear factor kappa B; Sevo: sevoflurane.

Treatment was given on cells by exposure to 100% oxygen or 0.5 MAC of sevoflurane in 60% oxygen for two hours starting at one hour after the addition of LPS ([Supplementary Figure 4](#)). A significant increase in TNF- α concentration in the cell culture supernatant at three hours after LPS stimulation (P < 0.05, [Supplementary Figure 4](#)) was inhibited by 100% oxygen or 0.5 MAC of sevoflurane in 60% oxygen starting at one hour after the addition of LPS (P < 0.01, **Figure 4A**). Furthermore, the LPS-induced nuclear translocation of the NF- κ B p65 subunit was prevented by 100% oxygen or 0.5 MAC of sevoflurane in 60% oxygen, as shown in **Figure 4B**. Classical inhibitor proteins in the NF- κ B signaling system consist of the single polypeptide I κ Bs: I κ B α , I κ B β and I κ B ϵ . In resting cells, I κ B binds and sequesters the NF- κ B dimer, and prevents DNA binding and transcriptional activation. The stimulus-responsive activation of I κ B kinase (IKK) results in the degradation of I κ Bs to release and activate NF- κ B [26]. In addition, the NF- κ B family is composed of five related transcription factors: p50, p52, p65 (also RelA), c-Rel, and RelB. In addition, RelB, c-Rel and p65 contain C-terminal transcription activation domains (TADs) that enable co-activator recruitment and target gene expression, especially NF- κ B/p65; and thus, mediate pro-inflammatory response [27]. Hence, we therefore performed an analysis of the NF- κ B signaling activation by measuring changes in the expression of phosphorylated p65, I κ B α and IKK α/β in total cellular proteins from LPS-stimulated cells treated with 100% oxygen or 0.5 MAC of sevoflurane in 60% oxygen. As shown in **Figure 4C**, LPS stimulated the increase in phosphorylated p65, I κ B α and IKK α/β expression, which were partly reversed by the treatment of 100% oxygen or 0.5 MAC of sevoflurane in 60% oxygen. The above findings suggest that both 100% oxygen and 0.5 MAC of sevoflurane in 60% oxy-

gen inhibit the activation of NF- κ B signaling in macrophages induced by LPS stimulation, contributing to the improvement of sepsis.

The 0.5 MAC of sevoflurane in 60% oxygen inhibited the activation of NF- κ B in human PBMCs stimulated by LPS or plasma obtained from septic patients

A clinical *in vitro* model of sepsis was induced in human PBMCs by LPS or plasma obtained from septic patients. This treatment was performed on cells through exposure to 100% oxygen or 0.5 MAC of sevoflurane in 60% oxygen for two hours starting at one hour after the challenge. A significant increase in TNF- α and IL-1 β concentration in the cell culture supernatant was observed at three hours after LPS stimulation, which were inhibited by 100% oxygen or 0.5 MAC of sevoflurane in 60% oxygen (P < 0.05; **Figure 5A** and **5B**). In addition, the nuclear translocation of the NF- κ B p65 subunit was also induced by LPS or plasma obtained from septic patients, which was blocked by 100% oxygen or 0.5 MAC of sevoflurane in 60% oxygen (**Figure 5C** and **5D**). These findings reveal that 0.5 MAC of sevoflurane in 60% oxygen reduces inflammation in human PBMCs through the inhibition of NF- κ B activation, and provides evidence of clinical safety for its protective effects on sepsis by 0.5 MAC of sevoflurane in 60% oxygen.

Discussion

Severe sepsis is a clinical continuum characterized by an acute infection associated with organ dysfunction, which often results in significantly poor outcomes and mortality. To date, studies using animal models of sepsis remain as part of the development of novel therapeutic agents. In the present study, we observed that

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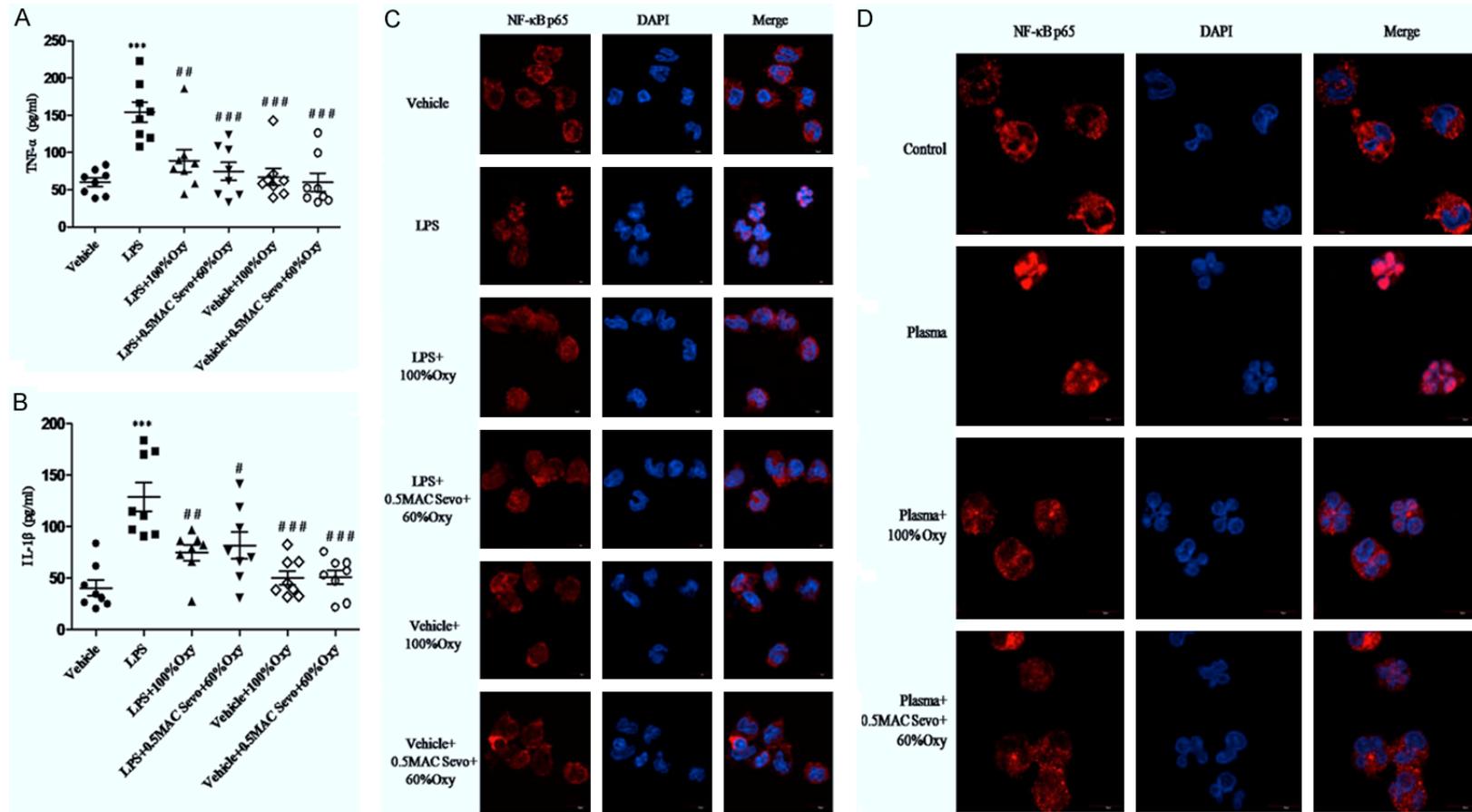


Figure 5. Treatment of 0.5 MAC sevoflurane in 60% oxygen protected human PMBCs against clinical in-vitro sepsis induced by LPS or plasma from septic patients through inhibition of NF-κB activation. Clinical in-vitro sepsis was induced in human PMBCs by 100 ng/ml LPS or the plasma from septic patients for 3 h. Cells were treated with 100% oxygen or 0.5 MAC sevoflurane in 60% oxygen for 2 h started at 1 h after the challenge. TNF-α (A) and IL-1β (B) concentrations were determined in cell culture supernatant from human PMBCs 3 h after LPS stimulation. Data are expressed as mean ± s.e.m. (n = 8 for each group). ****P* < 0.001 vs. vehicle group; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 vs. LPS group. Fluorescence images of NF-κB p65 subunit nuclear translocation captured on a confocal laser scanning microscope (×240) were got from human PMBCs 3 h after the stimulation with LPS (C) and with plasma from septic patients (D). h: hour; LPS: lipopolysaccharide; MAC: minimum alveolar concentration; NF-κB: nuclear factor kappa B; Oxy: oxygen; PMBC: Peripheral blood mononuclear cell; Sevo: sevoflurane.

the treatment with 0.5 MAC of sevoflurane in 60% oxygen was the best combination of oxygen and sevoflurane for protecting animals against lethality and lung injury induced by CLP or intraperitoneal injection of LPS, and there was a time window for its protective effects on LPS-induced sepsis. However, the administration of hyperoxia to clinic, especially in the intensive care unit, remains controversial. Calzia E et al. [8] reported in their review that although it has been well-established for more than a century that long-term exposure to pure oxygen results in central nervous oxygen toxicity, which induces potential harm to a lifesaver [28], several recent arguments support the use of ventilation with 100% oxygen, as a supportive measure during the first 12 to 24 hours of septic shock [11]. In addition, the use of hyperoxia is safe at least within the time frame of perioperative administration [29, 30]. Furthermore, the beneficial effects of the safe subtoxic regimens of normobaric hyperoxia were observed in various animal models of sepsis including gut-derived mouse sepsis [31], zymosan-induced mouse sterile sepsis [6], early hyperdynamic porcine fecal peritonitis [32] and CLP-induced rat sepsis [33]. In recent studies, it has also been demonstrated that administration of an anesthetic dose of sevoflurane and its metabolite reduces CLP-induced mortality [9, 10]. In addition, an anesthetic dose of isoflurane, which is the same type of anesthetic gas as sevoflurane, was reported to protect against lung injury and sepsis [34, 35]. Another recent research indicated that commonly used volatile anesthetic agents such as isoflurane and sevoflurane had a protective effect on vital organs, including lung insults during endotoxemia and generalized sepsis [36]. However, the use of an anesthetic dose of volatile anesthetics in experimental studies might have a severe consequence on mortality in sepsis [12]. In this study, we demonstrated for the first time that 0.5 MAC of sevoflurane in 60% oxygen was the best combination of oxygen and sevoflurane for providing significant protection against lethality and lung injury resulting from LPS- and CLP-induced sepsis.

Acute lung injury (ALI) secondary to sepsis is a complex syndrome associated with high morbidity and mortality. Nearly 50% of patients with severe sepsis will develop ALI, and in its more severe form, acute respiratory distress syndrome (ARDS) [37]. The respiratory system

is the most frequently affected organ system, and diffuse inflammation of lung parenchyma and severe lung dysfunctions are the first steps in the development of multiple organ failure and one of the leading causes of death in sepsis. In the present study, acute lung injury occurred to animals with CLP-induced sepsis characterized by increased lung inflammation and impaired alveolar endothelial and epithelial barriers. These were improved by the treatment of 0.5 MAC of sevoflurane in 60% oxygen. These results show that CLP led to significantly increased pathological scores for lung, lung MPO activity, the extent of neutrophil accumulation in the lung, lung wet-to-dry ratio, as well as increased protein production in the BALF, an indicator for assessing the extent of alveolar endothelial and epithelial barriers injury [38]. These were markedly improved through the treatment of 0.5 MAC of sevoflurane in 60% oxygen. In addition, the inhalation of 0.5 MAC of sevoflurane in 60% oxygen improved lung oxygenation in rats with CLP-induced sepsis, and further improved the survival rate animals with sepsis. In previous studies, Barth E and Hauser B *et al.* observed that ventilation with 100% oxygen markedly reduced pulmonary hypertension, as well as apoptotic death rate, in lungs during early hyperdynamic porcine fecal peritonitis [32]. Waisman D *et al.* provided evidences that safe subtoxic regimens of normobaric hyperoxia have the beneficial effects on pulmonary inflammatory response following CLP [33]. Sevoflurane anesthesia has also been proven to attenuate lung inflammation in rats with LPS-induced acute lung injury, and protect lung injury from a two-hit [39]. In a pig model of acute respiratory distress syndrome, sevoflurane at an end-tidal concentration of 1.5% was shown to ameliorate lung inflammatory response and improve oxygenation to a greater extent, compared to propofol [40]. The above statements further support the protective action against sepsis by 0.5 MAC of sevoflurane in 60% oxygen.

Based on literature, a dysregulated, excessive proinflammatory cytokine expression contributes to the pathogenesis of sepsis [41]. In the present study, abnormally increased cytokine/chemokine expression in serum and peritoneal lavage fluid (IL-1 β , IL-6, TNF- α , and HMGB1) occurred to animals with CLP-induced sepsis; which were inhibited by 0.5 MAC of sevoflurane in 60% oxygen. In addition, 0.5 MAC of sevoflu-

rane in 60% oxygen promoted the increase in IL-10 in serum and peritoneal lavage fluid from animals with CLP-induced sepsis, which was considered to be an anti-inflammatory factor [42, 43]. This was shown to benefit the improvement of sepsis, which was reported in a previous study [44]. Waisman D *et al.* provided evidences that support the beneficial effects of safe subtoxic regimens of normobaric hyperoxia on systemic and pulmonary inflammatory response following CLP [33]. Our previous study also revealed that inhaling 100% oxygen twice for two or three hours attenuates the increase in proinflammatory cytokine levels after zymosan injection [6]. In addition, there is growing evidence that volatile anesthetics, sevoflurane and isoflurane, have beneficial immunomodulatory effects on complex inflammation-mediated conditions [9, 45] and attenuate the sequestration of organ damage markers and inflammatory mediators including a broad variety of chemokines and cytokines in the initial hyperinflammatory phase of sepsis [9, 45]. These results suggest that 0.5 MAC of sevoflurane in 60% oxygen protect sepsis and its relevant lung injury may be through the reduction of inflammatory response on sepsis and the protection of alveolar endothelial and epithelial barriers; thus, improving sepsis and sepsis-induced lung injury.

Our results also revealed that both treatments of 100% oxygen and 0.5 MAC of sevoflurane in 60% oxygen protected LPS-induced *in vitro* sepsis by inhibiting NF- κ B activation, which plays a key role in regulating immune response to infection [25]. The inhibition to NF- κ B activation can reduce injury to organs (acute kidney injury, lung injury, and so on) resulting from sepsis [25, 46], and improve survival for critically ill patients [47]. These results indicate that the suppression of the hyperinflammatory phase in sepsis *via* the inhibition of the NF- κ B signaling pathway contributes to the protective effects on sepsis through 100% oxygen and 0.5 MAC of sevoflurane in 60% oxygen.

In order to explore the potential therapeutic actions of 0.5 MAC of sevoflurane in 60% oxygen or 100% oxygen in patients with sepsis, we examined the activation of NF- κ B in cells. Our results demonstrated that both treatments of 100% oxygen and 0.5 MAC of sevoflurane in 60% oxygen inhibited the nuclear translocation of NF- κ B from the cytoplasm to the nucleus in

human PBMCs induced by LPS stimulation or plasma from septic patients. These results suggest that 0.5 MAC of sevoflurane in 60% oxygen or 100% oxygen protected human PBMCs against clinical *in vitro* sepsis induced by LPS or plasma from septic patients that involved the NF- κ B signaling pathway. Thus, these results definitely provide some evidence for its treatment application in clinic, and further demonstrated that the immune/inflammatory mechanism plays an important role in its protective effects against sepsis through the treatment of 100% oxygen or 0.5 MAC of sevoflurane in 60% oxygen.

In summary, the administration of a subanesthetic dose of sevoflurane (0.5 MAC) in 60% oxygen effectively protects against sepsis, demonstrating that the combined administration of a subanesthetic dose of sevoflurane (0.5 MAC) with 60% oxygen is a safer, novel therapy for sepsis, without concerns of oxygen toxicity and the impact of an anesthetic dose of sevoflurane on mortality in sepsis. Hence, our data provides a potential therapy for sepsis in clinic.

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Disclosure of conflict of interest

None.

Authors' contribution

Erfei Zhang is in charge of primary experiment, Tian Shao, Zuoxu Hou and Zexin Zhang are in charge of *in vitro* experiment, Xiaoxia Wang, Bin Hu, and Wanwan Yang are in charge of instruction of analysis data. Yi Huang, Lize Xiong and Lichao Hou instruct the research.

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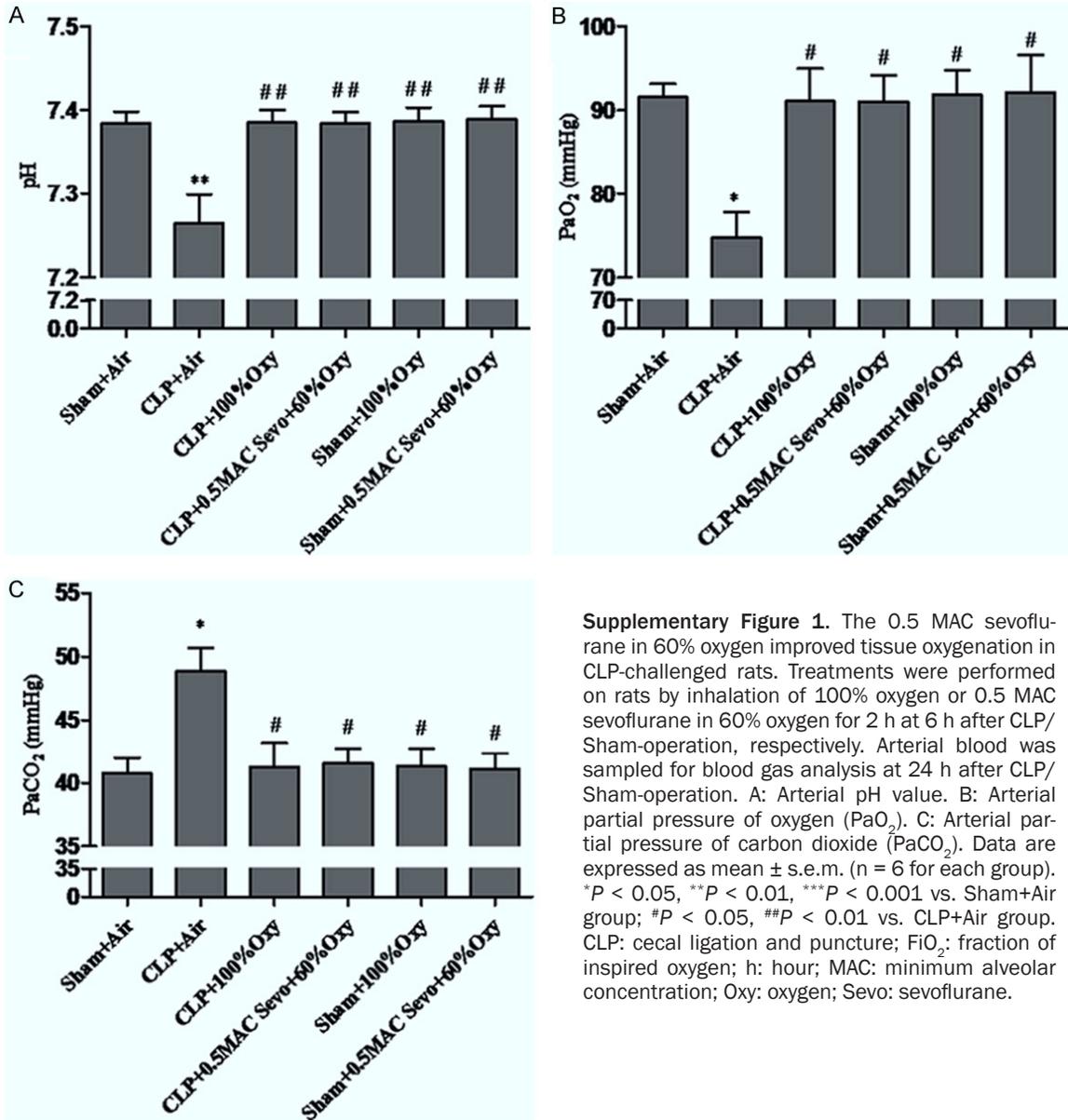
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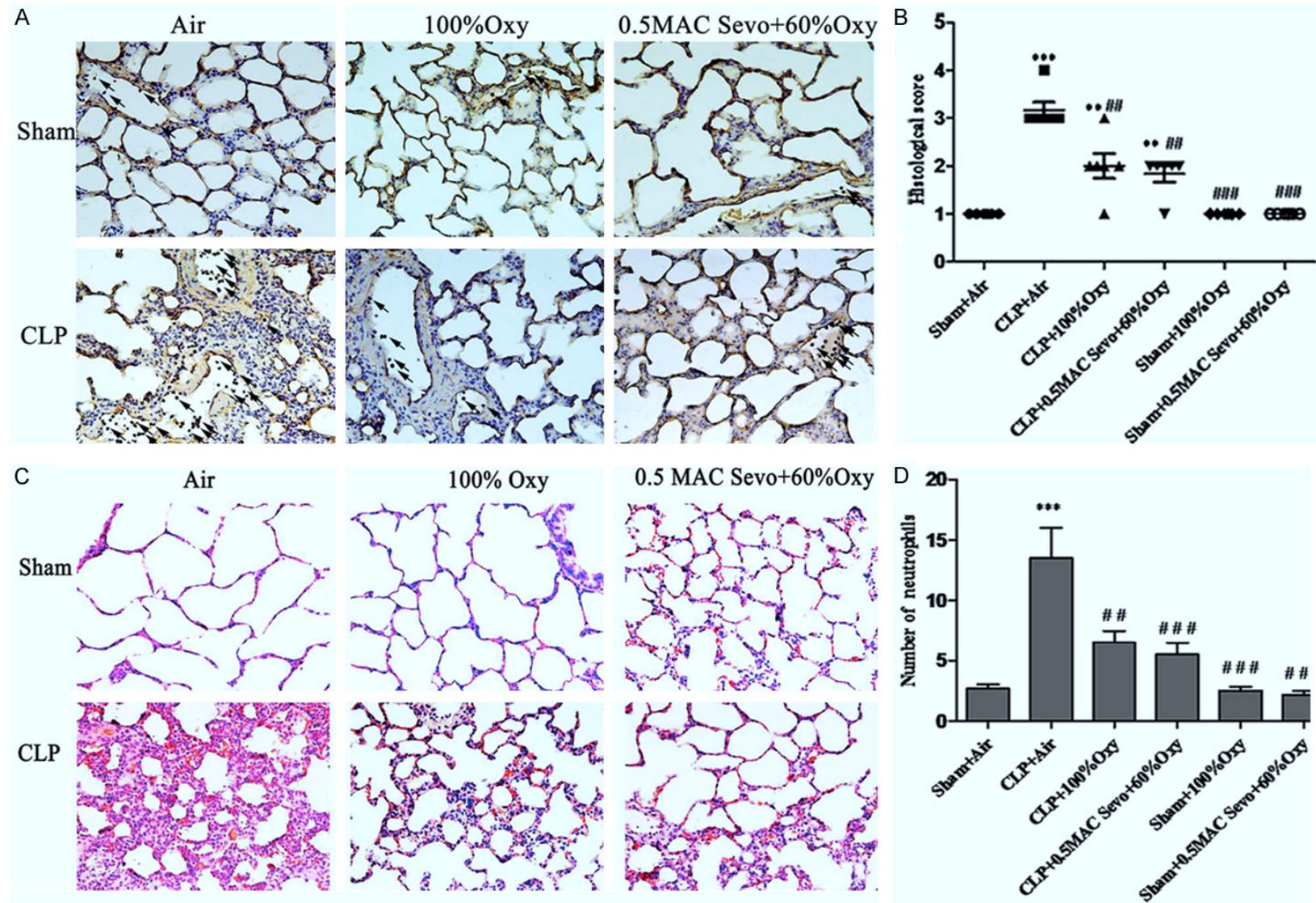
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Supplementary Figure 1. The 0.5 MAC sevoflurane in 60% oxygen improved tissue oxygenation in CLP-challenged rats. Treatments were performed on rats by inhalation of 100% oxygen or 0.5 MAC sevoflurane in 60% oxygen for 2 h at 6 h after CLP/Sham-operation, respectively. Arterial blood was sampled for blood gas analysis at 24 h after CLP/Sham-operation. A: Arterial pH value. B: Arterial partial pressure of oxygen (PaO₂). C: Arterial partial pressure of carbon dioxide (PaCO₂). Data are expressed as mean ± s.e.m. (n = 6 for each group). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. Sham+Air group; #*P* < 0.05, ##*P* < 0.01 vs. CLP+Air group. CLP: cecal ligation and puncture; FiO₂: fraction of inspired oxygen; h: hour; MAC: minimum alveolar concentration; Oxy: oxygen; Sevo: sevoflurane.

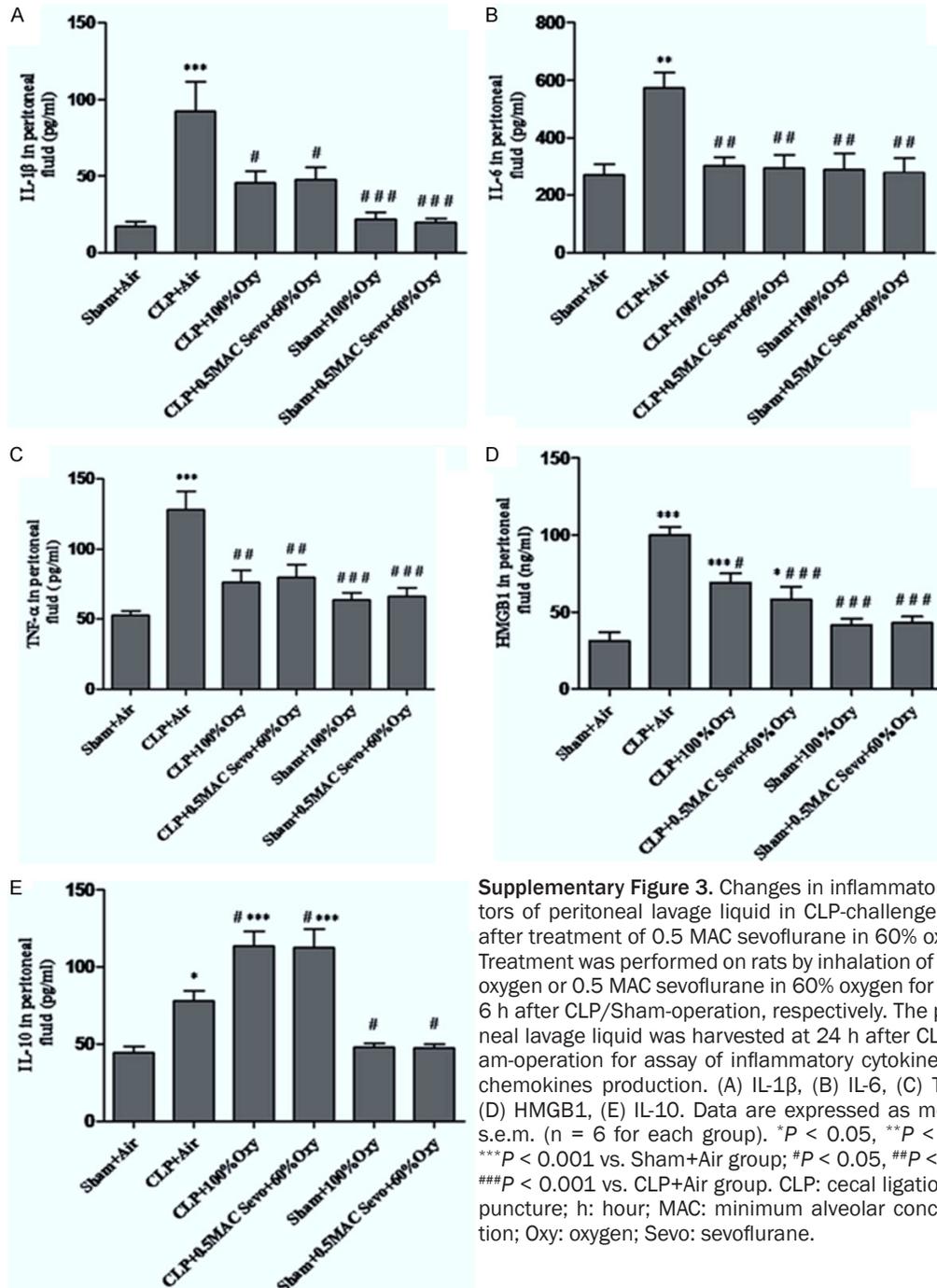
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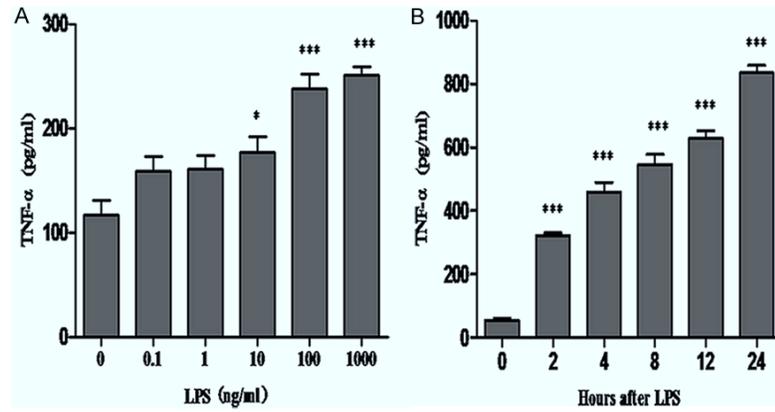
Supplementary Figure 2. The 0.5 MAC sevoflurane in 60% oxygen reduced lung pathological injury and neutrophil accumulation in CLP-challenged animals. CLP-induced sepsis was performed on SD rats. Treatment was performed on animals by inhalation of 100% oxygen or 0.5 MAC sevoflurane in 60% oxygen for 2 h at 6 h after the CLP/Sham-operation, respectively. A: The 0.5 MAC sevoflurane in 60% oxygen reduced lung pathological injury after CLP. For histological assessment of lungs in rats, lungs were harvested 24 h after CLP for histological observation under optical microscopy (original magnification: $\times 40$). Photomicrographs are representative of data obtained from lung sections derived from six animals. In Sham-operated animals, uniform and small alveoli were seen. While alveolar collapse,

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interstitial oedema, congestion, alveolar wall thickening, consolidation involving half of the lung and heterogeneous alveolar size were observed in animals from the CLP+Air group. Treatment of 100% oxygen or 0.5 MAC sevoflurane in 60% oxygen alleviated the above CLP-induced morphological changes in lung. B: The 0.5 MAC sevoflurane in 60% oxygen reduced pathological scores for lung in rats after CLP. Data are expressed as mean \pm s.e.m. (n = 6 for each group). C: The 0.5 MAC sevoflurane in 60% oxygen reduced neutrophil accumulation in lung after CLP. For assay of neutrophil accumulation, the lungs were harvested at 24 h after the CLP/Sham-operation, and were stained by immunohistochemistry using specific antibody reacted with neutrophils (arrows). Photomicrographs are representative of data obtained from lung sections derived from six animals (original magnification: $\times 40$). D: The 0.5 MAC sevoflurane in 60% oxygen inhibited increased number of neutrophil in lung after CLP (n = 6 for each group). Data are expressed as mean \pm s.e.m. (n = 6 for each group). $^{***}P < 0.01$, $^{****}P < 0.001$ vs. Sham+Air group; $^{##}P < 0.01$, $^{###}P < 0.001$ vs. CLP+Air group. CLP: cecal ligation and puncture; H&E: hematoxylin-eosin; h: hour; MAC: minimum alveolar concentration; Oxy: oxygen; Sevo: sevoflurane.



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Supplementary Figure 4. TNF- α concentrations in culture supernatant of RAW264.7 cells stimulated with LPS. A: Dose-dependent effects on TNF- α concentrations by LPS stimulation for 24 h. B: Time-dependent effects on TNF- α concentrations by stimulation of 100 ng/ml LPS. Data are expressed as mean \pm s.e.m. (n = 6 for each group). * P < 0.05, ** P < 0.01, *** P < 0.001 vs. vehicle group. h: hour; LPS: lipopolysaccharide; MAC: minimum alveolar concentration; NF- κ B: nuclear factor kappa B; Oxy: oxygen; Sevo: sevoflurane.