

Topotecan reduces sepsis-induced acute lung injury and decreases the inflammatory response via the inhibition of the NF- κ B signaling pathway

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Abstract

This study aims to determine the function of topotecan (TPT) in acute lung injury (ALI) induced by sepsis. The mouse sepsis model was constructed through cecal ligation and puncture (CLP). The ALI score and lung wet/dry (W/D) weight ratio were applied to evaluate the level of lung injury. Hematoxylin–eosin staining was used to examine the role of TPT in lung tissue in a CLP-induced ALI mouse model. Enzyme-linked immunosorbent assay and quantitative real-time polymerase chain reaction were used to detect the concentrations of inflammatory factors, such as interleukin-6 (IL-6), IL-1 β , and tumor necrosis factor- α . Western blot was used to detect relevant protein levels in the nuclear factor- κ B (NF- κ B) pathway. Moreover, 10-day survival was recorded by constructing the CLP model. The results indicated that TPT could improve lung tissue damage in mice and could significantly reduce lung injury scores ($p < 0.01$) and the W/D ratio ($p < 0.05$). Treatment with ammonium pyrrolidinedithiocarbamate obtained the similar results with the TPT treatment. Both significantly reduced the inflammatory response in the lungs, including reducing the number of neutrophils and total cells in the bronchoalveolar lavage fluid (BALF), significantly reducing the total protein concentration of the BALF, and significantly inhibiting the activity of MPO. Both also inhibited inflammatory cytokine expression and the levels of NF- κ B pathway proteins induced by sepsis. Furthermore, TPT significantly improved survival in sepsis. TPT improves ALI in

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the CLP model by inhibiting the NF- κ B pathway, preventing fatal inflammation.

KEYWORDS

acute lung injury, cecal ligation and puncture, inflammation, NF- κ B, topotecan

INTRODUCTION

Acute lung injury (ALI) has the characteristics of acute, diffuse, and inflammatory lung injury, which could increase alveolar-capillary permeability and lung weight.¹ ALI has a high mortality rate (35%–46%), with more severe lung injury at the onset being associated with a higher mortality rate.² Despite improvements in the treatment of ALI due to advancements in science and technology, the therapeutic effect of ALI still has limitations.³ At present, no effective drugs for the treatment of ALI have been developed. Therefore, it is necessary to identify novel pharmacological drugs for treating ALI.

Previous research found that topotecan (TPT), a topoisomerase 1 (Top1) inhibitor, can play an activating role in the transcriptional response to infection. The function of TPT is implemented through subsidiary Top1 transcriptional sensitization of pro-inflammatory factors.⁴ Previous studies have found that TPT could induce the secretion of risk-related molecules, thereby triggering cytokine production and dendritic cell activation.⁵ Previous studies have indicated that Top1 can reduce ventilator-induced lung injury and lipopolysaccharide-mediated ALI by inhibiting the nuclear factor- κ B (NF- κ B) pathway.⁶

Sepsis is defined as the occurrence of organ dysfunction when the host body is infected, which can be life-threatening in severe cases. At present, sepsis has become the leading cause of death worldwide.⁷ Approximately 30% of sepsis patients even develop multiple organ dysfunction syndromes (MODS).⁸ Moreover, previous studies have found that the lungs are relatively fragile organs and that 40% of ALI patients are caused by sepsis.⁹ The cecal ligation puncture (CLP) model is widely used in exploring the treatment of sepsis, such as in studying the impact of different treatments on the survival rate of patients with sepsis.¹⁰ However, the effect of TPT in the CLP model remains unknown.

This experimental study aimed to explore the function of TPT on ALI in a CLP-induced sepsis mouse model. A CLP model was constructed to verify the therapeutic effect of TPT on CLP-induced lung injury and to detect the effect of TPT on inflammatory cytokines. In addition, we explored the role of the NF- κ B pathway in the use of

TPT as a treatment for ALI induced by CLP. In the end, TPT was shown to attenuate the inflammatory response caused by lung injury, likely via the NF- κ B signaling pathway.

MATERIALS AND METHODS

Animals

The experiments in this study were approved by the ethics committee of our hospital and were performed according to the guidelines for Institutional Animal Care and User. C57BL/6 mice (20–22 g and 6–8 weeks old) were obtained from the Model Animal Research Institute of Nanjing University (Jiangsu, China). These mice were fed in the Animal Research Center of our hospital, which were housed under standard conditions (22 ± 2 °C, 40%–60% relative humidity, and 12-h light/dark cycle) for at least 7 days.

Model of sepsis

The mice were divided into four groups: sham group ($n = 8$), CLP group ($n = 8$), TPT + CLP group ($n = 8$), and CLP + ammonium pyrrolidinedithiocarbamate (PDTC) group ($n = 8$). We injected sodium pentobarbital (40 mg/kg body weight) intraperitoneally to anesthetize the mice. The CLP model was constructed as previously described.¹¹ The dose, concentration, and administration time of TPT and PDTC were determined based on correlational research¹² and our previous study.¹³ Furthermore, we used liquid nitrogen to snap-freeze the right lung and stored it at -80 °C, and the left lung was fixed with paraformaldehyde and embedded in paraffin for histological analysis.

Pulmonary histopathology and ALI score

We sliced the above-mentioned embedded paraffin tissue into 5- μ m sections. Hematoxylin–eosin (HE) staining was used to visualize the bone marrow, peripheral lymphatic organs, and lung tissues. The degree of lung

injury was assessed based on the standard reported in previous studies.¹⁴ The randomly selected visual fields were independently scored in a blind method. Then, we evaluated the scores of each parameter, including proteinaceous debris filling the airspaces, neutrophils in the interstitial space, neutrophils in the alveolar space, alveolar septal thickening, and hyaline membranes.

Bronchoalveolar lavage fluid (BALF) analysis

After the model was successfully constructed, we used the tracheal cannula to instill 1.8 ml of phosphate-buffered saline into the right lung and divided it into three instillations of 0.6 ml. We then slowly recovered the fluid and collected the BALF. In addition, we acutely centrifuged the BALF and collected the cells. The Diff-Quik stain solution was then used to stain the cells, which were counted through a light microscope. A bicinchoninic acid (BCA) protein assay kit (cat. no. 23227; Thermo Fisher Scientific) was used to examine the density of proteins in the BALF.

Detection of inflammatory factors

First, quantitative real-time-polymerase chain reaction (qRT-PCR) was used to detect the level of the inflammatory factors (interleukin-6 [IL-6], IL-1 β , and tumor necrosis factor [TNF]) at the messenger RNA (mRNA) level. The TRIzol reagent (Invitrogen) was used to extract the total RNA from the lung tissue. Then, complementary DNA was synthesized using a reverse transcription kit (Takara). Then, we performed RT-PCR on an ABI 7500 real-time PCR system (Applied Biosystems) using SYBR Premix Ex Taq II (Perfect Real-Time; TaKaRa). The primer sequences are detailed in Table 1. The $2^{-\Delta\Delta C_T}$ method was used to normalize the quantification of the target gene level. In addition, we used an enzyme-linked immunosorbent assay (ELISA) to examine the concentration of the inflammatory factors in the BALF based on the manufacturer's instructions.

TABLE 1 Primer sequences for real-time PCR

Targets	Forward primer (5'-3')	Reverse primer (5'-3')
TNF- α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
IL-1 β	GCAACTGTTCTGAACTCAACT	ATCTTTTGGGGTCCGTC AACT
IL-6	TAGTCCTTCTACCCCAATTTC	TTGGTCCTTAGCCACTCCTTC
β -Actin	GATCATTGCTCCTCTGAGC	ACTCTGCTTGCTGATCCAC

Abbreviations: IL, interleukin; PCR, polymerase chain reaction; TNF, tumor necrosis factor- α .

Evaluating lung edema using the wet/dry (W/D) weight ratio

After sacrificing the mouse, we immediately removed the left lung. A gauze was used to gently absorb moisture on the surface of the lungs, which were then weighed. Subsequently, we baked the lung tissue in an oven at 72°C for 48 h. Then, the lung tissue was removed and weighed; this was defined as the dry weight. We used the W/D weight ratio to evaluate lung edema.

Detection of myeloperoxidase (MPO) activity

We cut an appropriate amount of lung tissue and then added 1 ml of 0.5% cetyltrimethylammonium bromide for homogenization. Then, we examined the MPO activity using an MPO assay kit (Nanjing Jiancheng Co., Ltd.) based on the manufacturer's instructions.

Western blot analysis

We used lysis buffer, which contained phenylmethylsulfonyl fluoride (Roche), protease inhibitor cocktail (Roche), and the mammalian protein extraction reagent RIPA (Beyotime, China), to extract the total protein from the lung tissue. Then, the total concentration of the extracted protein was measured using the Bio-Rad protein detection kit (KeyGEN Biotech). A 30- μ g protein sample was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a 0.22- μ m polyvinylidene fluoride (Millipore) membrane, and incubated with specific antibodies (NF- κ B p105: total form, 1:1000, #13586, phosphorylated form, 1:1000, #4806; NF- κ B p65: total form, 1:1000, #8242, phosphorylated form, 1:000, #3033; and I κ B α : total form, 1:000, #4814, phosphorylated form, 1:1000, #4806 [Cell Signaling Technology]; GAPDH, 1:10,000, ab181602 [Abcam]). An ECL detection system (Tanon)

was used to examine the protein bands after incubating the secondary antibodies.

Survival study

A total of 80 mice were equally divided into the sham group ($n = 20$), CLP group ($n = 20$), CLP + TPT group ($n = 20$), and CLP + PDTC group ($n = 20$). The experimental procedure was conducted as described above. After the model was successfully constructed, the mice were kept in a standard environment with sufficient food and water. We then recorded and tested the 10-day survival rate of the mice.

Statistical analysis

The experiments were performed in triplicate. The SPSS 20.0 software (IBM) was used to perform statistical analysis on the data, which were presented as their mean \pm standard deviation (SD). Student's *t* test was used for comparisons of two groups, and comparisons of multiple groups were conducted via one-way analysis of variance through Fisher's least significant difference post hoc test. The Kaplan–Meier method was used to evaluate the survival rate among the sham group, CLP group, CLP + TPT group, and CLP + PDTC group, which was compared among these four groups using the log-rank test. Statistical significance was set at $p < 0.05$.

RESULTS

TPT protects mice against CLP-induced ALI and lung edema

To verify the safety effect of TPT, we used HE staining to visualize the tissue structure of the bone marrow and peripheral lymphatic organs after TPT treatment (Figure 1). We found that the bone marrow and peripheral lymphatic organs were not damaged by the TPT treatment.

H&E staining was also used to explore the function of TPT on lung tissue. Compared to the sham group, the lung tissue in the CLP group demonstrated bleeding, significant immune infiltration, and increased pulmonary edema, interstitial edema, and alveolar wall thickness (Figure 2a). In the CLP + TPT and CLP + PDTC groups, the lung tissues demonstrated marked improvement in effusion and edema. The lung injury scores were also evaluated using a blind method. Both the CLP + TPT and CLP + PDTC groups had markedly lower lung injury scores compared to the CLP group ($p < 0.05$; Figure 2b), suggesting that TPT might have significantly ameliorated the pathological lung tissue changes. The CLP group had large areas of lung tissue injury with local hemorrhage, whereas the CLP + TPT and CLP + PDTC groups had a lesser degree of injury and bleeding of the lung tissue (Figure 2c). The CLP group had a significantly increased lung W/D weight ratio compared to the control group

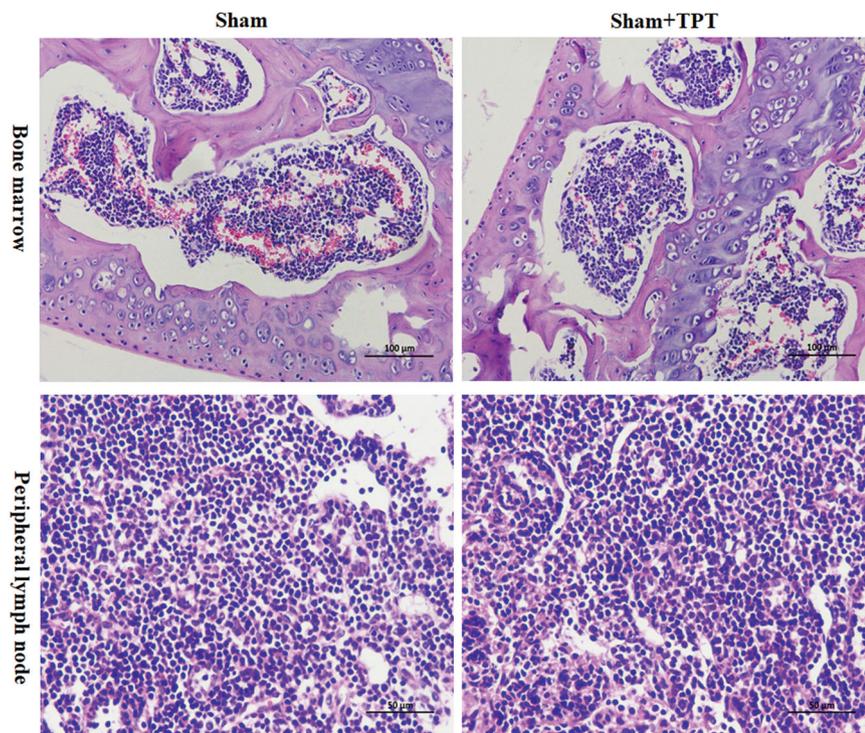


FIGURE 1 Bone marrow and peripheral lymphatic organs (hematoxylin and eosin stain). TPT, topotecan.

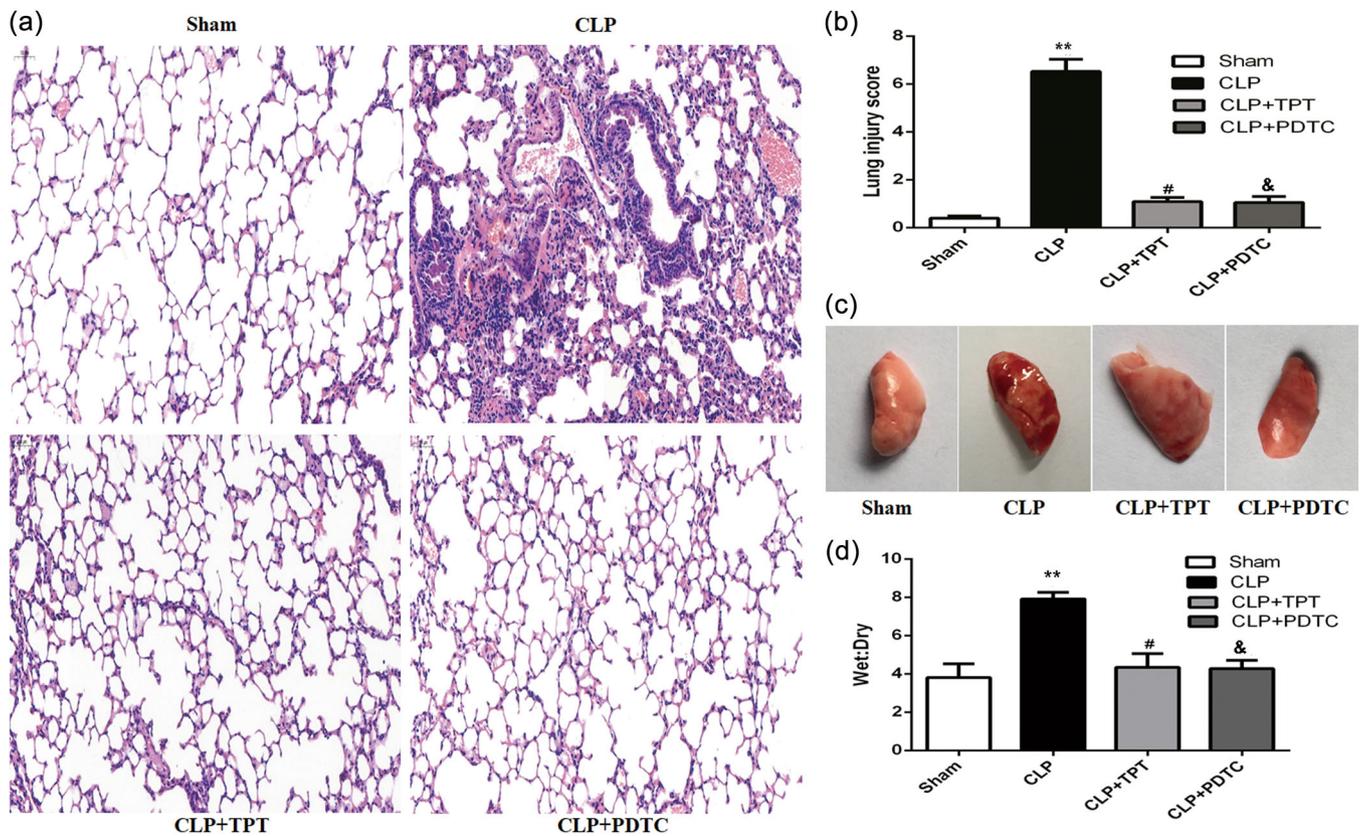


FIGURE 2 TPT ameliorates pulmonary inflammation and edema in acute lung injury induced by cecal ligation and puncture. (a) Hematoxylin and eosin staining for lung tissues in the sham, CLP, CLP + TPT, and CLP + PDTC groups. (b) Lung injury scores. (c) Lung injury in the harvested lung tissue. (d) Lung edema. ** $p < 0.01$: CLP group versus sham group. # $p < 0.05$: CLP + TPT group versus CLP group. & $p < 0.05$: CLP + PDTC group versus CLP group. ALI, acute lung injury; CLP, cecal ligation and puncture; PDTC, ammonium pyrrolidinedithiocarbamate; TPT, topotecan.

($p < 0.01$). In the TPT + CLP and CLP + PDTC groups, however, the lung W/D was markedly reduced compared to the CLP group ($p < 0.05$; Figure 2d). The above results all showed that treatment with TPT could significantly improve CLP-induced ALI.

TPT reduces the expression of inflammatory infiltration and activity of MPO

We examined the expression of inflammatory cells in the BALF to study the role of TPT on lung inflammation. In contrast to the sham group, the CLP group had significantly increased cell number and total protein concentration in the BALF ($p < 0.01$), whereas these were significantly lower in the CLP + TPT and CLP + PDTC groups ($p < 0.05$; Figure 3a, c). Neutrophil count and MPO activity were dramatically increased in the CLP model compared to the sham group ($p < 0.01$), but these decreased in the CLP + TPT and CLP + PDTC groups compared to the CLP group ($p < 0.05$; Figure 3b,d).

TPT reduced the expression level of inflammatory factors

We used qRT-PCR and ELISA to detect the expression levels of inflammatory factors in mRNA and protein in lung tissues across the different models. Compared to the sham group, the CLP group had significantly increased expression of inflammatory factors TNF- α , IL-6, and IL-1 β ($p < 0.01$; Figure 4). However, the TNF- α , IL-6, and IL-1 β levels were markedly lower in the CLP + TPT and CLP + PDTC groups than in the CLP group ($p < 0.05$). These results indicate that TPT could significantly reduce the inflammatory response in ALI induced by CLP.

TPT reduces the expression of the NF- κ B signaling pathway in ALI induced by CLP

The above results have found that TPT could reduce the production of inflammatory factors caused by CLP-induced ALI. To explore whether this effect was

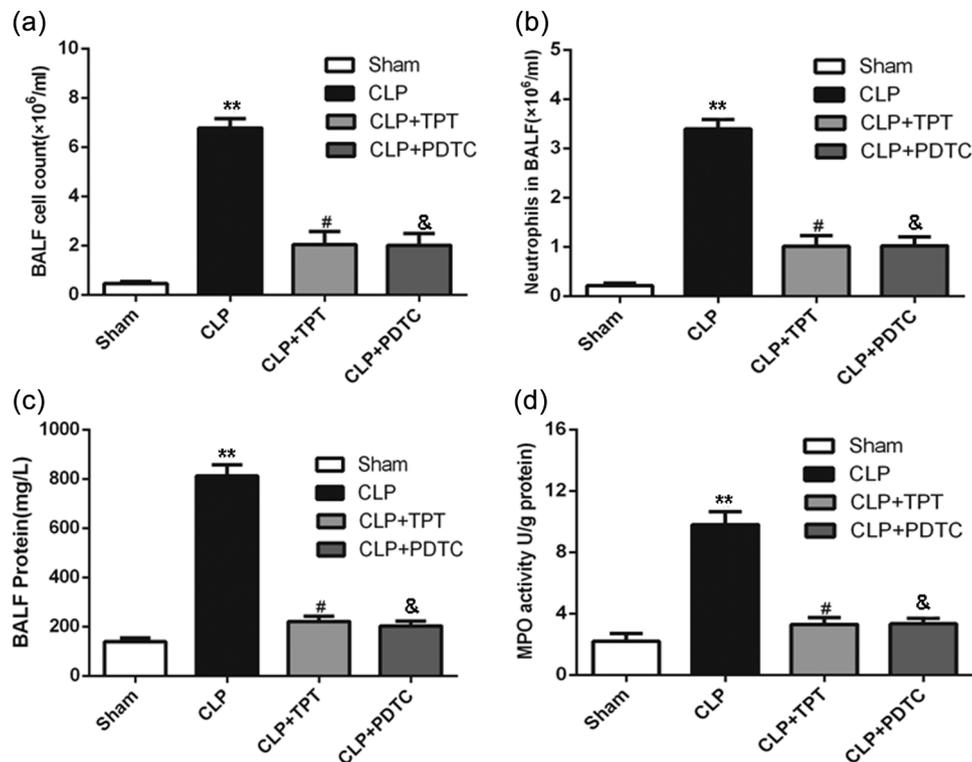


FIGURE 3 TPT reduces the expression of inflammatory infiltration and the activity of MPO. (a) BALF cell counts. (b) Neutrophils in the BALF were measured. (c) Lung permeability was evaluated through the BALF protein content. (d) MPO protein activity was measured. ** $p < 0.05$: CLP group versus sham group. # $p < 0.05$: CLP + TPT group versus CLP group. & $p < 0.05$: CLP + PDTC group versus CLP group. BALF, bronchoalveolar lavage fluid; CLP, cecal ligation and puncture; MPO, myeloperoxidase; PDTC, ammonium pyrrolidinedithiocarbamate; TPT, topotecan.

achieved through the NF- κ B signaling pathway, we used western blot to detect the expression levels of p65, p105, and I κ B α . The results are shown in Figure 5. Compared with the sham group, the CLP group had significantly increased p65, p105, and I κ B α levels ($p < 0.01$), but treatment with TPT and PDTC significantly reversed these ($p < 0.05$). The above results showed that the inhibition of the NF- κ B signaling pathway may play a key role in TPT reducing the inflammation caused by CLP-induced ALI.

Survival analysis

To further explore the effect of TPT on the treatment of CLP-induced ALI, we randomly divided the 60 mice into three groups and observed their 10-day survival period. The results are shown in Figure 6. Mice in the CLP + TPT and CLP + PDTC groups had significantly increased survival rates compared to those in the CLP group ($p < 0.05$). In summary, TPT was found to significantly improve the survival rate of CLP-induced ALI mice.

DISCUSSION

Sepsis can cause the dysfunction of various organs, among which lung tissue is the first to be damaged. In severe sepsis, acute respiratory distress syndrome can occur, which is the main cause of death in patients with a 30%–50% mortality rate.¹⁵ Therefore, there is an urgent need to explore new treatments for ALI to improve the prognosis of patients with severe sepsis.

TPT has demonstrated therapeutic effects in many diseases, such as leukemia, breast cancer, and small-cell lung cancer.¹⁶ The CLP model is a commonly used generic mouse model for humans with sepsis.¹⁷ In our study, we constructed a CLP model to assess the function of TPT on ALI induced by sepsis. We found that TPT suppressed the inflammatory response induced by CLP, reduced ALI, and decreased inflammatory cell infiltration, protein leakage, and the production of inflammatory factors. Furthermore, TPT significantly improved survival in sepsis. Similarly, Xu et al.¹⁸ found that TPT could inhibit the production of inflammatory factors induced by influenza and Ebola virus. Furthermore, other in vivo experiments have shown that TPT could prevent inflammation in mice.

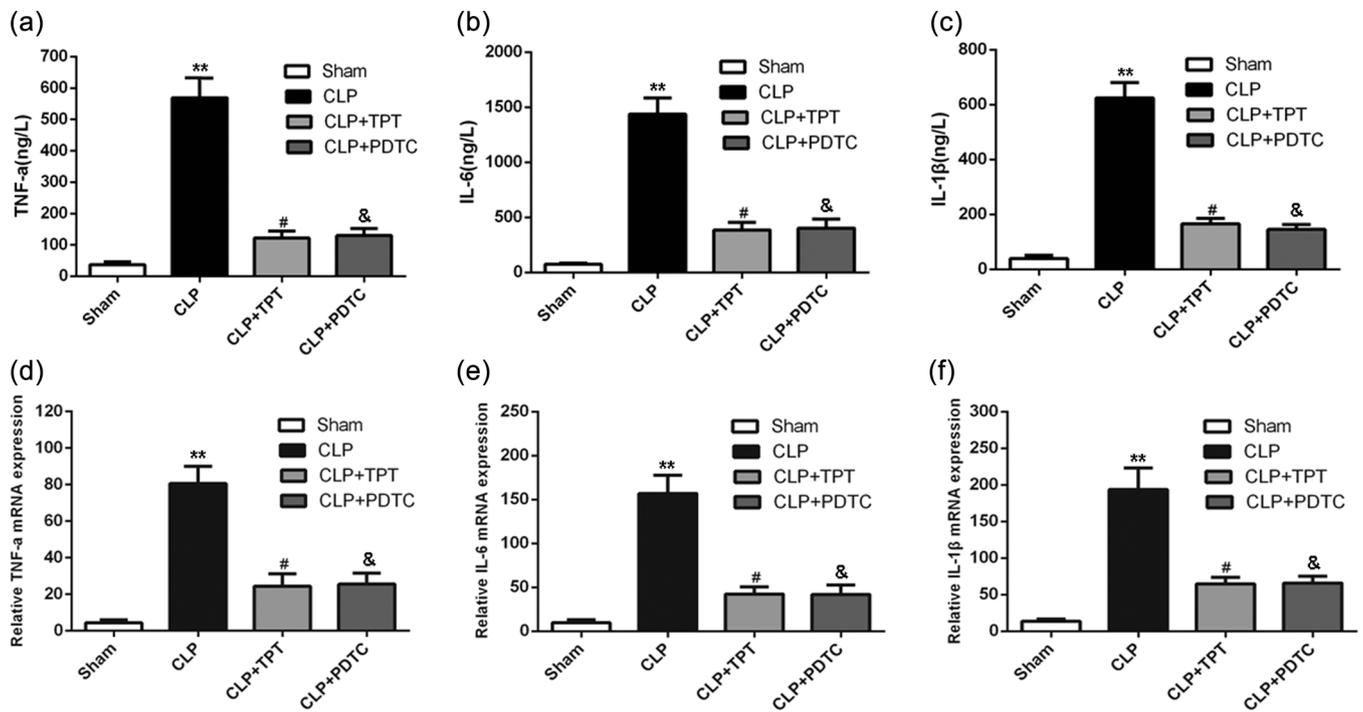


FIGURE 4 TPT decreases the inflammatory cytokine expression in the BALF of lung tissue in acute lung injury induced by cecal ligation and puncture. The mRNA expression of TNF- α , IL-6, and IL-1 β levels in the BALF (a-c) and their protein expression levels (d-f). ** $p < 0.01$: CLP group versus sham group. # $p < 0.05$: CLP + TPT group versus CLP group. & $p < 0.05$: CLP + PDTC group versus CLP group. BALF, bronchoalveolar lavage fluid; CLP, cecal ligation and puncture; IL, interleukin; mRNA, messenger RNA; PDTC, ammonium pyrrolidinedithiocarbamate; TNF, tumor necrosis factor; TPT, toptotecan;

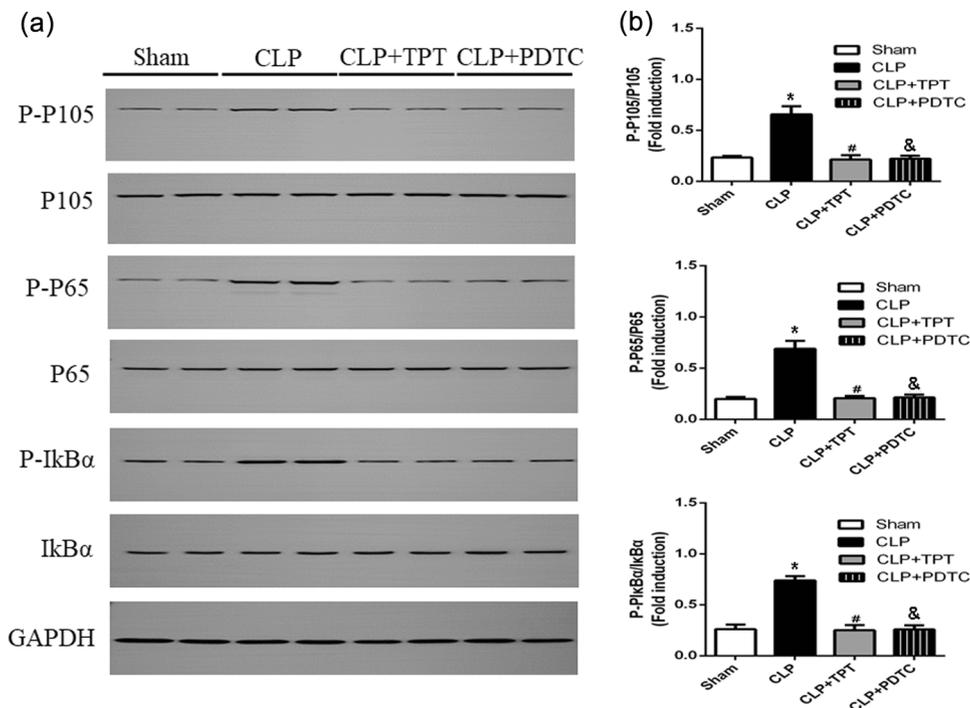


FIGURE 5 TPT inhibits the NF- κ B signaling pathway in acute lung injury induced by cecal ligation and puncture. (a) The protein expression of NF- κ B, including p-p105, p-p65, and p-I κ B. (b) The densitometric analysis for the protein expression levels of p-p105, p-p65, and p-I κ B. * $p < 0.05$: CLP group versus sham group. # $p < 0.05$: CLP + TPT group versus CLP group. & $p < 0.05$: CLP + PDTC group versus CLP group. CLP, cecal ligation and puncture; NF- κ B, nuclear factor- κ B; PDTC, ammonium pyrrolidinedithiocarbamate; TPT, toptotecan.

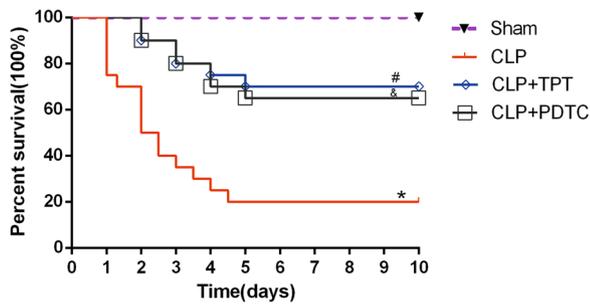


FIGURE 6 Survival analysis. The survival rates of the sham group, sham + CLP group, CLP group, and CLP + TPT group. * $p < 0.05$: CLP group versus sham group. # $p < 0.05$: CLP + TPT group versus CLP group. & $p < 0.05$: CLP + PDTC group versus CLP group. CLP, cecal ligation and puncture; PDTC, ammonium pyrrolidinedithiocarbamate; TPT, topotecan.

NF- κ B is one of the significant intracellular transcription factors widely spread for a variety of cells. Its activation plays a central part in the inflammatory response in sepsis, and this is the ultimate goal of the factors that precipitate sepsis. Furthermore, the gene expressions of a variety of cytokines, adhesion molecules, and chemotactic molecules are regulated by NF- κ B.¹⁹ Being one of the important signaling pathways toward the transcriptions and expressions of other inflammatory mediators, NF- κ B has important roles in the pathogenesis of many diseases.²⁰ Inhibition of NF- κ B expression could reduce the levels of lung macrophages and serum inflammatory factors in rats with severe trauma, as well as reduce inflammatory injury.²¹ On the other hand, activating NF- κ B could promote the production of inflammatory factors that could mediate the immune response and induce leukocytes to enter the lungs.²² Persistent organ dysfunction is also associated with continuous activation of NF- κ B,²³ whereas inhibiting NF- κ B might play a protective role in ALI.²⁴ Therefore, inhibiting the NF- κ B pathway could be therapeutic for ALI induced by sepsis. In our study, we found that treatment with TPT improved ALI induced by sepsis through the inhibition of the NF- κ B pathway. TPT also suppressed the production of inflammation factors (i.e., IL-6, IL-1 β , and TNF- α) in the BALF of lung tissue induced by CLP. TPT and PDTC dramatically inhibited the expression of p105, p65, and I κ B. Previous studies have cited the involvement of NF- κ B activation in regulating the occurrence and development of CLP-induced ALI.^{25,26} Thus, further study is needed to comprehensively explore the effect of TPT on the NF- κ B pathway in ALI induced by sepsis. In summary, TPT improved ALI caused by the CLP-induced activation of NF- κ B.

At present, no other studies have discussed the role of TPT in ALI. In our research, we found that TPT can effectively protect against CLP-induced ALI. Our research results are consistent with the study conducted by Rialdi.²⁶ In other words, TPT may become a new drug for the treatment of ALI. Therefore, it is necessary to further explore the clinical application of TPT.

CONCLUSIONS

TPT can attenuate CLP-induced ALI. Inhibiting the expression of the NF- κ B signaling pathway could possibly play a key role in reducing the inflammatory response of TPT. Therefore, it is necessary to conduct further research on the clinical application of TPT.

AUTHOR CONTRIBUTIONS

Xiaoxia Wang, Tianxiang Xu, Jiajia Jin, Yong Song, Tangfeng Lv, and Mei Gong were responsible for the conception and design of the research and drafting of the manuscript. Xiaoxia Wang, Tianxiang Xu, Bing Wan, Lingxiao Bai, Yong Song, and Tangfeng Lv performed the data acquisition, data analysis, and interpretation. Xiaoxia Wang, Tianxiang Xu, Ting Gao, and Jiajia Jin participated in the design of the study and performed the statistical analysis. All authors read and approved the final manuscript. The authors contributed equally to this study.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ETHICS STATEMENT

Not applicable.

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