Activated protein C inhibits lung injury induced by LPS via downregulating MAPK signaling

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Abstract. The aim of the present study was to investigate the effect and the underlying mechanism of activated protein C (APC) in lipopolysaccharide (LPS) induced lung injury, as well as the potential mechanism. According to the treatment, 50 rats were randomly divided into 5 groups: Control, model (LPS), low-dose group [LPS + 0.1 mg/kg recombined human activated protein C (rhAPC)], median-dose group (LPS + 0.3 mg/kg rhAPC) and high-dose group (LPS + 0.5 mg/kg rhAPC). Then, inflammation in the lung was assessed using hematoxylin and eosin (H&E) staining. Following the collection of bronchoalveolar lavage fluid (BALF), the number of leukocytes and neutrophils in BALF was counted, and superoxide dismutase (SOD) activity was assessed, as well as the expression levels of interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α using ELISA. Subsequently, the expression and phosphorylation of P-38, extracellular signal-regulated kinase (Erk)-1/2, and c-Jun N-terminal kinase (JNK) were estimated using western blotting. Based on H&E staining, rhAPC markedly suppressed inflammatory infiltration in the lung induced by LPS in a dose-dependent manner. In addition, rhAPC also significantly attenuated the accumulation of leptocytes and neutrophils, and the reduction of SOD in BALF induced by LPS in a dose-dependent manner. rhAPC also significantly attenuated the elevation of IL-1β, IL-6 and TNF-α in BALF induced by LPS in a dose-dependent manner. Further mechanistic analysis revealed that rhAPC treatment could evidently attenuate the phosphorylation levels of P-38, Erk1/2 and JNK in the lung induced by LPS in a dose-dependent manner. In conclusion, APC significantly alleviated the lung inflammation induced by LPS by downregulating the phosphorylation of P-38, ERK1/2 and JNK.

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Introduction

Acute lung injury (ALI) is a common syndrome in the clinic characterized by an abnormity of hypoxemia, epithelial integrity, non-cardiogenic lung edema, neutrophil and leukocyte accumulation, and an intense inflammatory response in lung (1). Despite of the development of medical technologies and medicines, ALI remains the leading cause of morbidity and mortality in critically ill patients (2). Thus, it is of importance to further explore the mechanism of ALI. Numerous causes are considered to contribute to ALI, including trauma, pneumonia, acid aspiration, and sepsis (3). Among them, bacterial infection is one of the most important inducer for sepsis. Lipopolysaccharide (LPS), a component of Gram negative bacterial cell membrane, is considered to play an important role in inflammatory response and immune dysfunction (4), but the mechanism of LPS-induced ALI is not fully elucidated.

Considering of the aforementioned information, multiple researches have been conducted to explore the mechanism of LPS-induced ALI, as well as potential therapies. Jiang *et al* (4) have identified that trillin can exert a protective effect on LPS-induced ALI via regulating the Nrf2/NF-κB signaling pathway. Lee *et al* (5), have demonstrated that 1-hexadecyl-3-(trifluoroethyl)-sn-glycero-2-phosphomethanol (MJ33) can serve as an inhibitor for NADPH oxidase (type 2) to against ALI associated inflammation. Besides, Do-Umehara *et al* (6), have documented that transcription factor Miz1 inhibits the expression of C/EBP-δ to suppress the inflammation during ALI.

Activated protein C (APC) is reported to enhance autophagy with rapamycin against sepsis-induced ALI (7). Moreover, APC prevents LPS-induced pulmonary vascular injury via attenuating the expression of cytokine (8). Inhaled APC protects mice from ventilator-induced lung injury via inhibiting the activation of cytosolic phospholipase A2 (9). In addition, increased APC mediates acute traumatic coagulopathy in mice (10). However, the detailed mechanism of APC in lung injury remains fully understood. In the present study, the potential mechanism of APC in ALI pathogenesis was identified, so that could provide a deeper understanding, or new insights for LPS-induced ALI.

Materials and methods

Lung injury animal model. This animal experimental protocol was authorized by the Ethics Committee of Southeast

University Affiliated Zhongda Hospital (Jiangsu, China). Adult female Sprague Dawley rats weighted 280-320 g (n=50) aged 8~10 weeks were purchased from the Shanghai Laboratory Animal Research Center (Shanghai, China) to apply for the following research of this study. Rats were housed in a SPF condition at a temperature of 22-24˚C and humidity of 40-70% with a 12 h light/dark cycles, and kept with free access to food and water. After adapted for 1 week, rats were utilized to construct a lung injury using LPS as previously described (11). Briefly, rats were randomly divided into five groups with 10 mice in each: i) Control group (saline), ii) model group (LPS), iii) low-dose group (LPS + 0.1 mg/Kg recombined human APC (rhAPC, Xigris; Eli Lilly Nederland BV, Houten, The Netherlands)), iv) median-dose group (LPS + 0.3 mg/Kg rhAPC), and v) high-dose group (LPS + 0.5 mg/Kg rhAPC). Rats were administered intravenously with saline or rhAPC. At 15 min after treatment, rats were anesthetized with 3% of chloral hydrate, and then intratracheally administrated with 20 μg of LPS dissolved in 50 μl of phosphate buffer saline (PBS) to induce ALI. For the control group, equal volume of PBS was used to instead of LPS solution in the process of lung injury.

Isolation of Bronchoalveolar lavage. After treated with LPS for 6 h, rats were anesthetized and sacrificed. Followed by this, bronchoalveolar lavage was collected using 0.5 ml of sterile PBS for 3 times (total volume=1.5 ml) to obtain the bronchoalveolar lavage fluid (BALF). Then, total leukocyte count and neutrophil count were estimated using a hemocytometer (Qiujing, Shanghai, China). Subsequently, BALF samples were centrifuged at 1,500 rpm at 4˚C for 10 min, and superoxide dismutase (SOD) activity of the supernatants was measured using a test kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China) according to manufacturers' protocol.

Analysis of inflammatory cytokines contained in BALF. The expression levels of IL-1β (catalogue no.: RLB00), IL-6 (catalogue no.: R6000B), and tumor necrosis factor (TNF)-α (catalogue no.: RTA00) were detected using enzyme-linked immune sorbent assay according to manufacture's protocols (R&D Systems, Inc., Minneapolis, MN, USA). All the experiments were conducted in triplicate, and mean value of them was computed as the final result.

Hematoxylin & eosin (H&E) staining. To estimate the inflammation in lung tissue, H&E staining was conducted for paraffin embedded sections. Briefly, the right lungs were removed at the end of the experiment, and parts of tissues were dehydrated using decreasing concentrations of ethanol, embedded in paraffin wax, and cut into slices with thick of 5 μm. Then, slices were deparaffinized, and rehydrated in decreasing concentrations of ethanol. Sections were heated for 3 min at 110˚C in 10 mmol/l Tris/1 mmol/l EDTA (pH 9.0) antigen retrieval buffer followed by 10 min at 95˚C and then cooled to 20˚C.

Western blot analysis. Lung tissues were homogenized in a 5 volumes of pre-cold lysis buffer (BioSource International Inc.; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing protease inhibitor cocktail (0.01%; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Then, tissue samples were centrifuged at 12,000 g at 4˚C for 10 min. Followed by this, the supernatants were collected, and the concentrations were determined using the BCA method (Thermo Fisher Scientific, Inc.). Then, supernatants were boiled with equal volume of loading buffer for 10 min. Total 10 μg protein was loaded into 12% SDS-PAGE gel, and transferred electrophoretically on a PVDF membrane. After blocked with 5% skim milk, blots were incubated with specific antibodies (p38 (catalogue no.: 8690), p-p38 (4511), ERK1/2 (9194), p-ERK1/2 (9101), c-Jun N-terminal kinase (JNK) (9252), and p-JNK(9255)) purchased from Cell Signaling Technology, Inc., (Danvers, MA, USA). After washing, blots were incubated with horseradish peroxidase-conjugated second antibodies. Subsequently, blots were washed, and visualized using ECL-detection system (PerkinElmer, Inc., Waltham, MA, USA).

Statistical analyses. In the present study, GraphPad Prism v6.0 software (GraphPad Software, Inc., La Jolla, CA, USA) was applied for statistical analyses. Continuous data was presented as mean ± standard deviation (SD). Comparison among groups was estimated using one-way analysis of variance followed by multiple comparisons using the least significant difference post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of rhAPC on lung injury induced by LPS. After rats sacrificed, the morphologic changes of lung tissues in different groups were estimated using H&E staining. The staining results showed that a larger number of neutrophil infiltration was around the lung vessel and airway, and distributed in the alveolar and interstitial in the LPS group compared with the control group. After pre-treated with rhAPC, the infiltration of inflammatory cells were obviously reduced, indicating that rhAPC could relieve the inflammatory level in lung injury tissues caused by LPS (Fig. 1).

Variations of Leukocyte, neutrophil, and SOD levels in BALF. The leukocytes and neutrophil counts, and SOD activity in BALF were also investigated in the present study. The counting results showed that the number of leukocyte was significantly higher in the LPS group than in control group, and rhAPC treatment could significantly decrease the number of leukocyte with a dose-dependent manner ($P<0.01$; Fig. 2A). Meanwhile, the number of neutrophil in BALF was also remarkably elevated in the LPS group compared with the control group, and rhAPC also could obviously decrease the number of neutrophil in BALF with a dose-dependent manner ($P<0.05$, Fig. 2B). However, the SOD activity level was significantly decreased in the LPS group compared with the control group, and rhAPC could evidently abort this elevation with a dose-dependent manner ($P<0.05$, Fig. 2C).

Variations of inflammatory cytokines in BALF. Meanwhile, inflammatory cytokines, including IL-1β, IL-6, and TNF-α, were also investigated in LPS induced lung injury. The detection showed that the expression level of IL-1β was evidently increased in the LPS group compared with the control group, but rhARC could significant abort this change with a

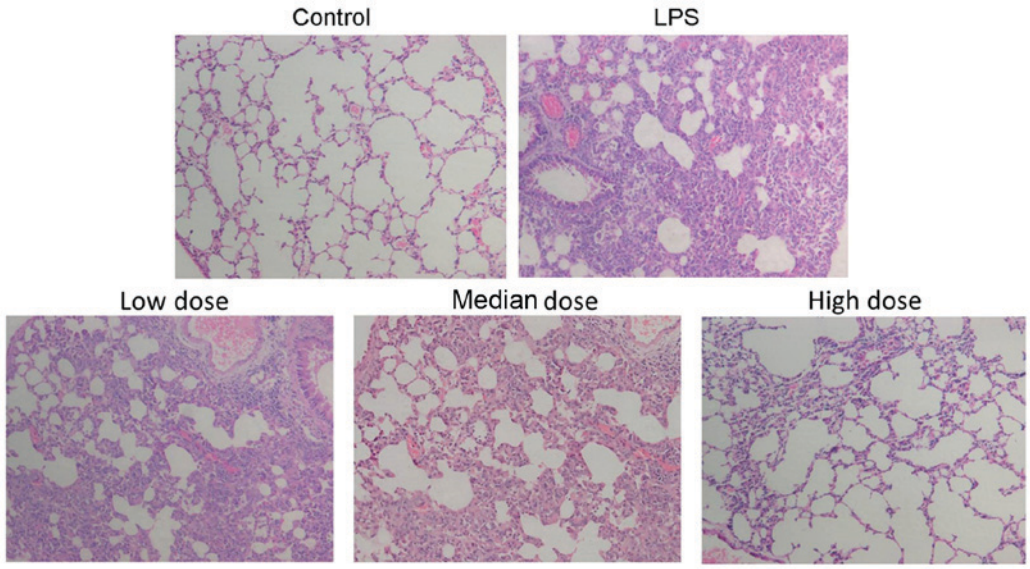

Figure 1. Infiltration of inflammatory cells in lung tissues determined using H&E staining (magnification, x200). The treatments applied for the different groups were as follows: Control group (saline), model group (LPS), low-dose group (LPS + 0.1 mg/kg rhAPC), median-dose group (LPS + 0.3 mg/kg rhAPC) and high-dose group (LPS + 0.5 mg/kg rhAPC). H&E, hematoxylin and eosin; LPS, lipopolysaccharide; rhAPC, recombined human activated protein C.

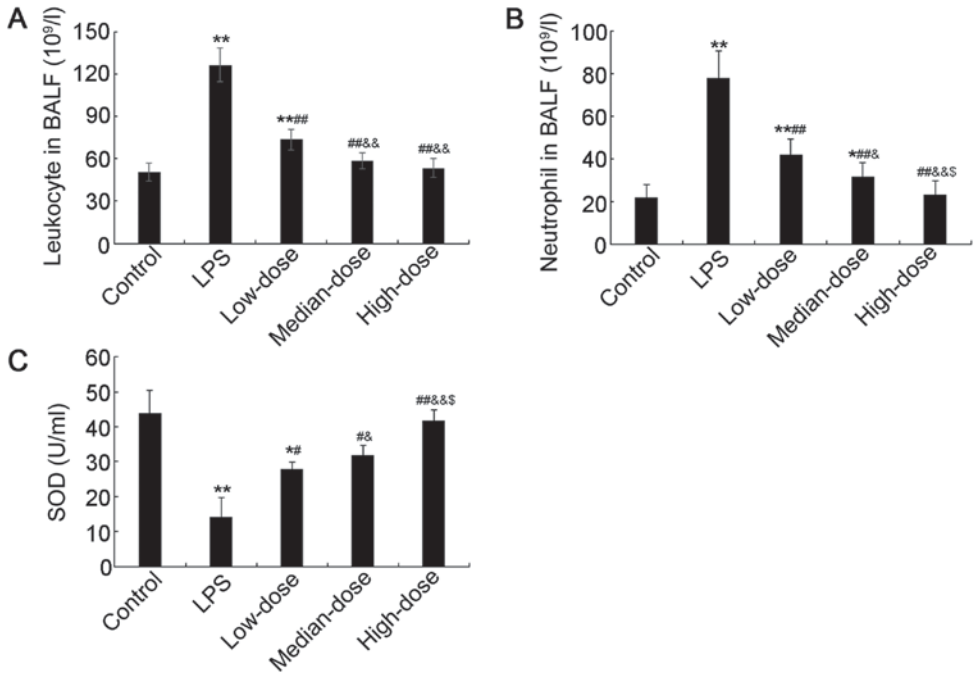

Figure 2. Leukocyte and neutrophil number, and SOD activity in BALF. (A) Leukocyte number in BALF; (B) neutrophil number in BALF; and (C) SOD activity in BALF. The treatments applied for the different groups were as follows: Control group (saline), model group (LPS), low-dose group (LPS + 0.1 mg/kg rhAPC), median-dose group (LPS + 0.3 mg/kg rhAPC) and high-dose group (LPS + 0.5 mg/kg rhAPC). *P<0.05 and **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. LPS; $^{\&}$P<0.05 and $^{\&\&}$P<0.01 vs. Low-dose group; $^{\$}$P<0.05 vs. Median-dose group. SOD, superoxide dismutase; BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharide; rhAPC, recombined human activated protein C.

dose-dependent manner (P<0.05; Fig. 3A). Meanwhile, the expression levels of IL-6 and TNF-α were also elevated in LPS-treated group than in the control group, and pre-treated with rhAPC could significantly inhibit these elevation with a dose-dependent manner (P<0.05; Fig. 3B and C), which were consistent with the trend in IL-1β.

Pathway of rhAPC involved in lung injury. To further investigate the mechanism of rhAPC, expression and phosphorylation of participators involved in the MAPK signaling pathway, including P-38, Erk1/2, and JNK were determined. The results showed that there were no significantly differences identified in the expression levels of P-38, Erk1/2, and JNK in LPS and rhAPC treated groups compared with the control group, but the phosphorylation levels of P-38, Erk1/2, and JNK were significantly up-regulated in LPS treated group, and rhAPC could significantly attenuate these elevations with a dose-dependent manner (Fig. 4).

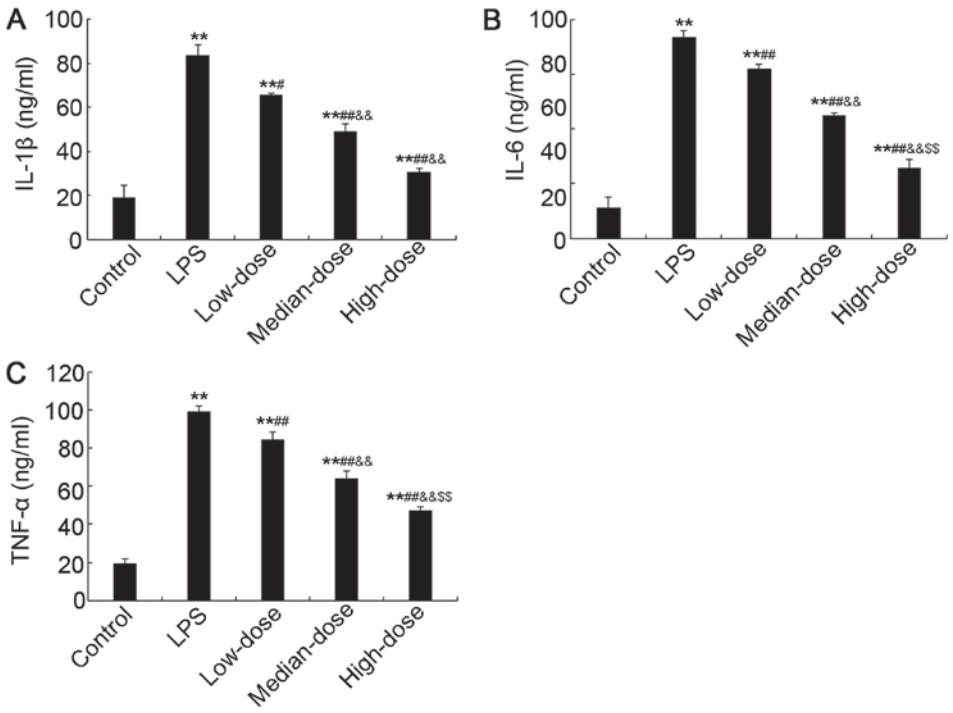

Figure 3. Expression of IL-1β, IL-6 and TNF-α in BALF determined using ELISA. Expression of (A) IL-1β, (B) IL-6 and (C) TNF-α. The treatments applied for the different groups were as follows: Control group (saline), model group (LPS), low-dose group (LPS + 0.1 mg/kg rhAPC), median-dose group (LPS + 0.3 mg/kg rhAPC) and high-dose group (LPS + 0.5 mg/kg rhAPC). **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. LPS; $^{\&\&}$P<0.01 vs. Low-dose group; $^{\$\$}$P<0.01 vs. Median-dose group. IL, interleukin; TNF, tumor necrosis factor; BALF, bronchoalveolar lavage fluid; LPS, lipopolysaccharide; rhAPC, recombined human activated protein C.

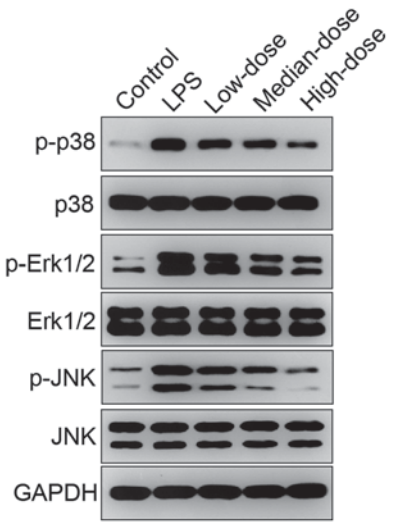

Figure 4. Expression and phosphorylation levels of P-38, Erk1/2 and JNK in lung tissues determined using western blotting. The treatments applied for the different groups were as follows: Control group (saline), model group (LPS), low-dose group (LPS + 0.1 mg/kg rhAPC), median-dose group (LPS + 0.3 mg/kg rhAPC) and high-dose group (LPS + 0.5 mg/kg rhAPC). Erk, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; rhAPC, recombined human activated protein C; p-, phosphorylated.

Discussion

In the present study, based on a LPS-induced ALI model, the mechanism of rhAPC in the regulation of ALI associated inflammation was investigated. The results showed that rhAPC could significantly attenuate the accumulation and infiltration of inflammatory cells, as well as the inflammatory cytokines, including IL-1β, IL-6, and TNF-α induced by LPS with a dose-dependent manner. Meanwhile, rhAPC also could evidently reverse the reduction of SOD activity level caused by LPS with a dependent manner. Further investigation showed that the phosphorylation of P-38, Erk1/2, and JNK might involve in the process of rhAPC against ALI associated inflammatory response.

As aforementioned, LPS is a common pathogen for the occurrence of ALI (12). Several researchers have identified that LPS can significantly up-regulated the accumulation of neutrophil and mononuclear leukocytes, and the expression levels of cytokines, including TNF-α, IL-1β, and IL-6 (13-15). In the present study, significant elevations were also identified in the recruitment of neutrophil and mononuclear leukocytes and the expression of TNF-α, IL-1β, and IL-6, indicating that ALI model had been successfully induced. Meanwhile, the SOD activity was significantly reduced in LPS-treated group compared with the control group. Oxidative stress is commonly identified in ALI and acute respiratory disease syndrome (16), and SOD is an important approach to revise the anomality of oxidative stress (17,18). Thus, a significant reduction of SOD activity might further contribute to the development of the inflammation during ALI. Further analysis showed that LPS could significantly increase the phosphorylation of P-38, Erk1/2, and JNK, indicating that LPS might drive the inflammation in ALI via MAPK signaling pathway. Park *et al* (19) have identified that mitochondrial ROS regulates MAPK and NF-κB signaling pathways to participate in the regulation of LPS-induced pro-inflammatory response in microglia. He *et al* (20) have

documented that Baicalein attenuates inflammatory response induced by LPS via suppressing TLR4 mediated NF-κB signaling pathway. These findings indicated that LPS might enhance the inflammatory response via regulating MAPK signaling pathway.

APC, a serine protease, is considered to play an important role in the maintenance of hemostasis (21), inhibitions of cytokines release, and reduction of leucocyte recruitment (22). In the past years, several studies have recognized that APC has an anti-inflammatory effect that is beneficial for stroke (23), sepsis (24), ischemia-reperfusion injury (25) in humans. In the present study, APC was identified to significantly attenuate the accumulations and infiltrations of leukocyte and neutrophil, and the release of cytokines, including TNF-α, IL-6 and IL-1β, as well as the reduction of SOD activity, in LPS-induced ALI with a dose-dependent manner. These findings confirmed that APC indeed performed a protective role against the increased inflammation induced by LPS in the lung. Nick *et al* (26), have found that recombinant human activated protein C could attenuate human endotoxin-induced lung inflammation via inhibiting neutrophil chemotaxis, which was consistent with the identification observed in the present study. However, the mechanism of this process is still unclear. In the present study, APC was identified to evidently decrease the phosphorylation levels of P-38, Erk1/2, and JNK induced by LPS with a dose-dependent manner, indicating APC could attenuate the inflammation induced by LPS via MAPK signaling pathway. Shi *et al* (27), have confirmed that geniposide can suppress LPS-induced inflammation by inhibiting NF-κB, MAPK and AP-1 signaling pathway. Meanwhile, Liang *et al* (28), have also observed that thymol attenuates LPS-induced inflammation via down-regulating NF-κB and MAPK signaling pathways. All of these findings indicated that inhibition of MAPK signaling pathway might perform a crucial role in suppressing inflammation induced by LPS. Considering these identification, it is supposed that APC also conduct a protective effect against LPS-induced ALI via MAPK signaling pathway, but the cross talk between MAPK and NF-κB was still needed to be further investigated.

In conclusion, APC could significantly attenuate the increase infiltrations and accumulations of leukocyte and neutrophil, releases of IL-1β, IL-6, and TNF-α, and reduction of SOD activity in LPS-induced ALI with a dose-dependent manner via the MAPK signaling pathway. However, the cross talk between MAPK and other potential signaling pathways was still needed further exploration.

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Availability of data and materials

All data generated and analyzed during this study were included in this published article.

Authors' contributions

JZ conducted the majority of the experiments and wrote the manuscript. RH, SJ and XX collected and analyzed the data. WT designed the study and approved the final manuscript for submission. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal experimental protocol was authorized by the Ethics Committee of Southeast University Affiliated Zhongda Hospital (Jiangsu, China).

Consent for publication

Not applicable.

Competing interests

All of the authors have no conflict of interest in this research.

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