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Through modulating SIRT3, theacrine reduces inflammation and lung damage in septic mice.

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Abstract

The goal of this study is to determine how theacrine affects the development of sepsis and the severity of lung damage caused by sepsis. **Methods:** The mice were given injections of 3 mg/kg LPS dissolved in 50 μ L PBS or only PBS (control group = 10), creating a lung damage model of lipopolysaccharide (LPS)-induced sepsis. One hour after LPS treatment, theacrine was orally gavaged to the other three groups of mice (ten mice per group) at doses of 10, 20, and 40 mg/kg, respectively. The impact of theacrine on lung damage was confirmed by hematoxylin and eosin (H&E) staining. We measured oxidative stress and inflammatory factors using enzyme-linked immunosorbent assays (ELISA) and quantitative polymerase chain reactions (qPCR). The impact on apoptosis and mechanism of action were confirmed using TUNEL and immunoblot tests. **Results:** In mice with LPS-induced sepsis, theacrine had a significant impact on lung injury and lung relief score ($p < 0.01$). It also reversed the increased levels of inflammatory cytokines in a dose-dependent manner ($p < 0.01$). Furthermore, theacrine significantly reduced the intensity of increased levels of ROS and MDA, and improved the levels of SOD in lung tissues ($p < 0.01$). In addition, it reduced lung damage and enhanced LPS-induced cell death in the lungs via activating the SIRT3 pathway ($p < 0.01$). **Results:** Theacrine reduces inflammation and lung damage in septic mice by modulating SIRT3, suggesting it may be a promising therapeutic development lead for sepsis-related lung injury and inflammation. **Tags:** SIRT3 pathway, oxidative stress, sepsis, lung damage, theacrine

INTRODUCTION

Sepsis is defined as an organ dysfunction caused by an aberration in the host's response to infection [1]. Nearly 30 % of patients with sepsis develop multiple organ dysfunction syndrome. The lungs are one of the most vulnerable organs to sepsis [2]. Lipopolysaccharide (LPS) could induce acute inflammation by stimulating host cells to produce inflammatory cytokines and also induce acute lung injury (ALI) by recruiting activated neutrophils as well as macrophages into the lungs [3,4]. Drug therapy is very important for the treatment of sepsis patients and to improve the therapeutic effect, there is a need to

develop a large number of effective therapeutic drugs to improve the cure rate [5-7]. SIRT3 is an NAD-dependent deacetylase mainly confined to the mitochondria and its deficiency is related to the regulation of mitochondrial function and redox homeostasis [8]. Previously, it has been reported that expression of SIRT3 is reduced in LPS-induced acute lung injury and SIRT3 deficiency also aggravates LPS-induced inflammation and ROS production, making acute lung injury more serious [9]. So far, studies have shown

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that multiple drugs target SIRT3 and significantly improve the treatment effect of sepsis. *Camellia assamica* var *kucha* is a unique plant, mainly distributed in provinces of China [10]. Theacrine (1,3,7,9-tetramethyluric acid), one of the major alkaloids found in *Camellia assamica* var *kucha*, is a purine alkaloid associated with bioactive functions such as anti-depressant, sedative, hypnotic and lipid metabolism regulation [11,12]. In addition, it has anti-inflammatory and antioxidant effects as it has been shown to reduce inflammation, and oxidative stress and improve liver damage [13,14]. Furthermore, studies have revealed that Theacrine activates SIRT3 [14]. However, the role and mechanism of Theacrine in sepsis-induced lung injury remain unclear. This study aims to investigate the effect of Theacrine in a sepsis model and to elucidate its potential as a drug for the treatment of sepsis.

EXPERIMENTAL

Animals

Male C57BL/6 mice (40) were obtained from Shanghai Laboratory Animal Center (Shanghai, China). The experiment was approved by the Ethics Committee of Southwest Medical University (approval no. Ky2018029). Animals were handled in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines [15]. Mice were injected with 3 mg/kg LPS (Sigma, USA) dissolved in 50 μ L PBS or only sterile PBS (control group = 10). The remaining three groups (divided into ten mice per group) were administered theacrine (10, 20, or 40 mg/kg), (purchased from Sigma) by oral gavage 1 hour after LPS treatment.

Histological analysis

The lungs of mice were recovered after sacrificing the mice. Lung tissues were fixed with 5 % PFA, then embedded with paraffin and cut into slices. The sections were counterstained with hematoxylin-eosin staining (H&E). Lung injury score was determined by considering hemorrhage and alveolar wall thickness. Staining degree was quantified as follows: 0, 1, 2, 3 and 4 representing no staining, mild staining, moderate staining, severe staining and very severe staining, respectively.

Quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from lung tissues using RNA purification Kit (Tiangen, Beijing, China) and reverse transcribed to generate cDNA according to the manufacturer's specifications. The primers are listed in Table 1.

Enzyme-linked immunosorbent assay (ELISA)

The IL-1 β , TNF- α , IL-6, MDA, and SOD levels were assayed by ELISA kit (Abcam, UK). Samples were aspirated into wells and biotin-conjugated primary antibodies (1:1000) were added into the wells before the addition of avidin-conjugated HRP for 2 hours at room temperature. Then, enzyme substrate was added for color development and the result was measured with a microplate reader (Varioskan LUX, Thermo, USA) at 450 nm wavelength.

Immunofluorescence

Lung tissues were fixed with 5 % PFA. Afterward, sections were sliced and incubated with the ROS kit or TUNEL kit (Beyotime, Beijing, China). After rinsing with PBS and counterstaining with DAPI, the images were captured under a fluorescence microscope (Carl Zeiss, Germany).

Western blotting

Lung tissue lysates were collected after RIPA buffer addition. Protein concentration was obtained with a BCA protein assay kit (P0010, Beyotime, China).

Table 1: Primers used for qPCR

Gene	Forward primers (5' - 3')	Reverse primers (5' - 3')
TNF- α	ATGAGCACAGAAAGCATGATCC	ACAAGCAGGAATGAGAAGGG
IL-6	TGGATGGTCTTGGCTTAGCC	ACTGATGCTGGTGACAACCAG
IL-1 β	GAGCACCTTCTTTTCTTCATCTT	TCACACACCAGCAGGTTATCATC
β -actin	ATCTACGAGGGCTATGCTCTCC	CTGATCCACATCTGCTGGAAGG

Then proteins were separated with 10 % SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Then the membranes were incubated with 5 % BSA followed by primary monoclonal antibodies including Bax (1:1000, ab32503, Abcam, UK), Cleaved caspase 3 (1:1000, ab32402, Abcam, UK) and GAPDH (1:1000, ab8245, Abcam, UK) for 2 h at room temperature. The membranes were maintained in HRP secondary



antibodies for 2 h (1:2000) at room temperature and the signals were detected with ECL detection kit.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Data are presented as mean \pm standard deviation (SD). $P < 0.05$ was considered statistically significant.

RESULTS

Theacrine relieves lung injury induced by LPS

The results of the histological examination of LPS-induced acute lung injury and relief score of theacrine administration are displayed in Figure 1. The mice displayed tissue injury characterized by irregular alveolar integrity. This phenotype was reversed after the administration of theacrine at a concentration of 10, 20 and 40 mg/kg. Administration of 20 and 40 mg/kg of theacrine significantly improved the lung score ($p < 0.05$).

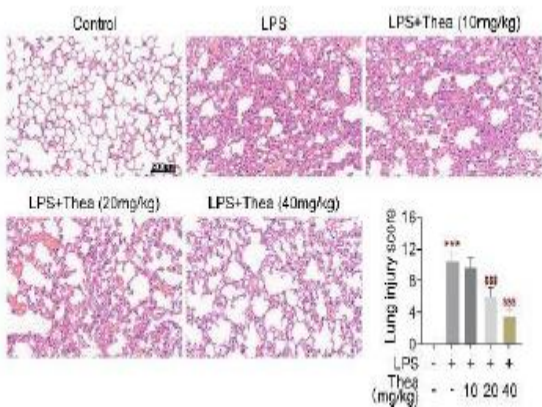


Figure 1: Histological analysis of lung tissues and injury score in control, LPS and theacrine-treated LPS mice at various concentrations. *** $P < 0.001$, LPS vs control; \$\$\$ $p < 0.001$, LPS + theacrine vs LPS

Theacrine inhibits inflammatory cytokines

The effect of theacrine on inflammatory markers in LPS-induced mice is shown in Figure 2. Administration of LPS significantly increased the levels of IL-6, IL-1 β and TNF- α levels ($p < 0.05$). However, theacrine significantly reversed the increased levels of these cytokines in a dose-dependent manner ($p < 0.05$; Figure 2 A).

Furthermore, this trend was confirmed by the results of ELISA (Figure 2 B). Therefore, theacrine inhibits the production of inflammatory cytokines in LPS-induced mice.

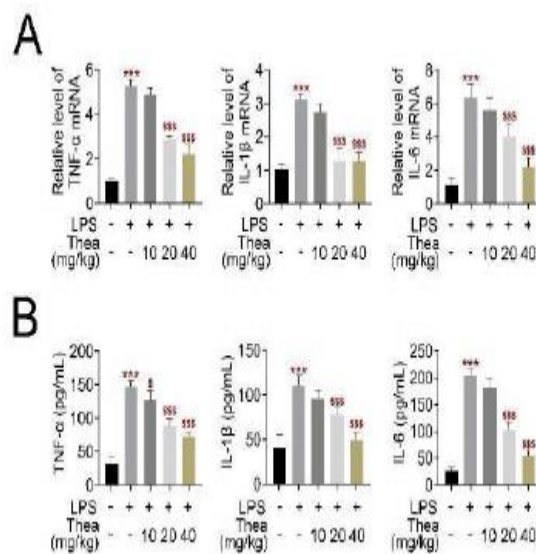


Figure 2: Theacrine inhibited inflammatory cytokines. (A) mRNA levels of IL-6, IL-1 β and TNF- α in lung tissues of control, LPS and theacrine-treated LPS-induced mice at the indicated concentrations of theacrine; (B) Levels of IL-6, IL-1 β and TNF- α in lung tissues of control, LPS and theacrine-treated LPS-induced mice at the indicated concentration of theacrine. *** $P < 0.001$, LPS vs control; \$ $p < 0.05$, \$\$\$ $p < 0.001$, LPS + theacrine vs LPS

Theacrine alleviates LPS-induced oxidative stress

The effect of Theacrine on LPS-induced oxidative stress was explored in the mice model. The levels of reactive oxygen species were determined by immunostaining experiment. LPS treatment increased the ROS levels in the lung tissues of mice (Figure 3 A). However, Theacrine treatment at a concentration of 10, 20 and 40 mg/kg reversed the increased intensity of ROS caused by LPS induction in lung tissues. Furthermore, Theacrine treatment significantly reduced the LPS-induced increase in the MDA levels and significantly improved the levels of SOD ($p < 0.05$; Figure 3 B). Therefore, Theacrine alleviates LPS-induced oxidative stress in lung tissues.

Theacrine improved LPS-induced apoptosis of lung tissues

The results of TUNEL assays revealed that LPS treatment induced apoptosis in lung tissues (Figure 4 A). However, Theacrine suppressed apoptosis of lung tissues caused by LPS treatment (Figure 4 A). In addition, LPS treatment increased Bax as well as cleaved caspase-3 expression in the lung tissues, whereas Theacrine treatment suppressed apoptosis of lung tissues, with the decreased expression of Bax as well as cleaved caspase-3 (Figure 4 B). Therefore, Theacrine improved LPS-induced apoptosis of lung tissues.

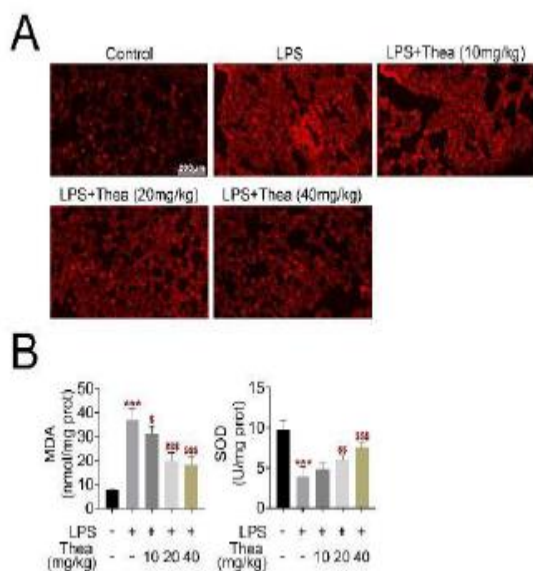


Figure 3: Theacrine alleviated LPS-induced oxidative stress. (A) ROS levels in lung tissues of control, LPS and theacrine -treated LPS-induced mice; (B) Levels of MDA and SOD in lung tissues of control, LPS and theacrine-treated LPS-induced mice. *** $P < 0.001$, LPS vs control; \$ $p < 0.05$, \$\$ $p < 0.01$, \$\$\$ $p < 0.001$, LPS + theacrine vs LPS

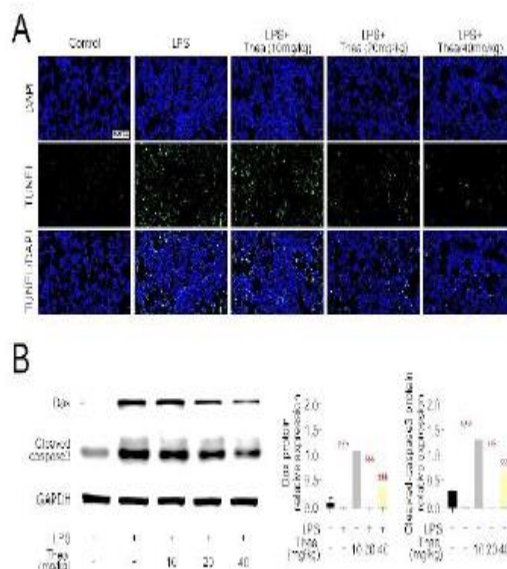


Figure 4: Theacrine improved LPS-induced apoptosis of lung tissues (A) Apoptosis levels in lung tissues of control, LPS and theacrine-treated LPS mice; (B) Expression levels of Bax and cleaved caspase 3 in lung tissues of control, LPS and theacrine-treated LPS mice. *** $p < 0.001$, LPS vs control; \$\$\$ $p < 0.001$, LPS + theacrine vs LPS

Theacrine alleviated lung injury by activating SIRT3 pathway

The level of expression of SIRT3 protein in the lung tissues of LPS-induced mice is shown in Figure 5. LPS significantly reduced the expression of SIRT3 protein in the lung tissues ($p < 0.05$). However, theacrine treatment reversed the increased SIRT3 expression caused by LPS treatment in lung tissues (Figure 5 A). The effects of theacrine-mediated lung injury were analyzed by an examination of the SIRT3 inhibitor, 3-TYP. As shown in Figure 5 B, 3-TYP stimulation increased the cytokine levels, which was suppressed by theacrine. The decreased MDA as well as SOD levels induced by theacrine was reversed by 3-TYP treatment (Figure 5 C). In addition, 3-TYP treatment induced the expression of Bax as well as cleaved caspase 3 in lung tissues, effectively reversing the effect of theacrine treatment (Figure 5 D). Taken together, theacrine alleviated lung injury by activating the SIRT3 pathway.

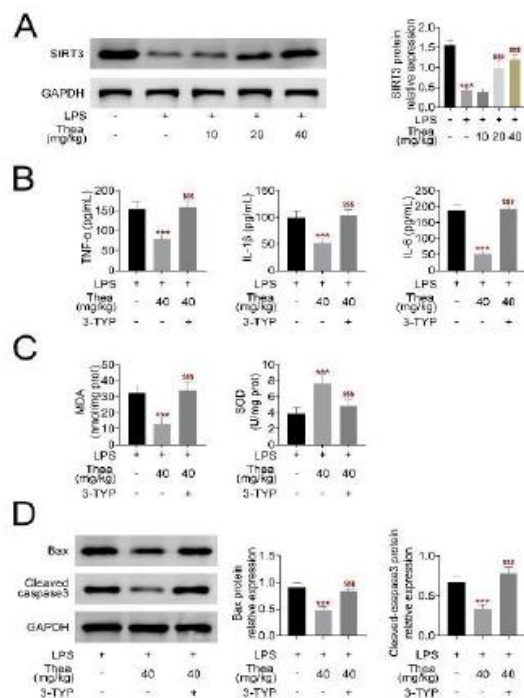


Figure 5: Theacrine alleviated lung injury by activating SIRT3 pathway. (A) Expression levels of SIRT3 in lung tissues; (B) Levels of IL-6, IL-1 β and TNF- α in lung tissues; (C) MDA and SOD levels in lung tissues; (D). Expression levels of Bax and cleaved caspase 3 in lung tissues. *** $p < 0.001$, LPS vs control; \$\$\$ $p < 0.001$, LPS + theacrine vs LPS

DISCUSSION

Sepsis is a clinical process of systemic inflammation caused by bacterial infection. It is a common complication in critically ill patients after surgery and is the basis for multiple organ improvement disorder syndrome. The lung is the most affected organ in multiple organ injury complicated by sepsis. Acute lung injury (ALI) appears earliest and has the highest incidence [16]. It is a systemic inflammatory response syndrome which is caused by various internal and external pulmonary pathogenic factors [16]. In sepsis, various inflammatory factors are activated, which lead to impaired immune function and damaged lung tissues, and this promotes the occurrence and development of ALI [17]. Although there has been some progress in the clinical application of drugs to treat it, there is still a need to develop more effective therapeutic drugs.

Theacrine has been shown to possess good physiological activities such as anti-depressant, sedative and hypnotic, anti-inflammatory and analgesic properties, reduces the stress damage of liver cells and is one of the research hotspots of purine alkaloids [18]. In addition, it strengthens the heart, and diuresis, dilates coronary artery and relaxes bronchial smooth muscles [18]. Theacrine has been shown to ameliorate liver fibrosis in rats via the activation of the Sirtuin 3-farnesoid X receptor pathway [12]. Interestingly, theacrine has been reported to alleviate sepsis-induced acute kidney injury by suppressing the NLRP3/caspase-1 inflammasome [20]. In this study, an LPS-induced sepsis mice model was recruited and used to induce lung injury. Theacrine was found to improve lung injury, oxidative stress and apoptosis caused by sepsis.

Studies have shown that damage to lung tissue caused by oxidative stress plays an important role in sepsis-induced ALI. Many factors can induce the body to produce free radicals and lead to the homeostasis disorder, so that free radicals cannot be smoothly removed, resulting in lung tissue damage and ultimately leading to ALI [19]. Therefore, avoiding oxidative stress in ALI is crucial to relieving symptoms [19]. In this study, Theacrine was shown to also alleviate sepsis-induced ALI via the suppression of oxidative stress.

Previously, it was shown that SIRT3 expression is reduced in LPS-induced ALI and that SIRT3 deficiency aggravates LPS-induced inflammation and ROS production, making ALI more serious [9]. Therefore, SIRT3 could serve as a target for LPS-induced ALI. Interestingly, the result of this study shows that theacrine activates SIRT3 and improves LPS-induced lung tissue injury. However, the precise mechanism needs further study.

CONCLUSION

Theacrine activates SIRT3 pathway and improves LPS-induced lung injury, inflammation, oxidative stress and apoptosis in sepsis. Therefore, it can potentially be developed as a drug for the treatment of sepsis and related inflammatory responses.

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