Rosavin inhibits neutrophil extracellular traps formation to ameliorate sepsis-induced lung injury by regulating the MAPK pathway

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Abstract

Background: Sepsis is a systemic organ dysfunction caused by infection, and the most affected organ is the lungs. Rosavin, a traditional Tibetan medicine, exerts an impressive anti-inflammatory effect. However, its effects on sepsis-related lung damage have not been investigated.

Purpose: This study aimed to investigate the effects of Rosavin on cecal ligation and puncture (CLP)-induced lung injury.

Methods: The sepsis mouse model was established by CLP, and the mice were pretreated with Rosavin to explore whether it contributed to the alleviation of lung injury. Hematoxylin-eosin (H&E) stain and lung injury score were used to assess the severity of lung injury. The bronchoalveolar lavage fluid (BALF) inflammatory mediators (tumor necrosis factor-\(\alpha\) [TNF-\(\alpha\)], interleukin-6 [IL-6], IL-1\(\beta\), and IL-17A) were detected by ELISA. The number of neutrophils in BALF was detected using flow cytometry. The immunofluorescence assay was used to detect histone and myeloperoxidase (MPO) in lung tissues. Then, the western blot was performed to detect the expression of mitogen-activated protein kinase (MAPK) pathways (extracellular regulated kinase [ERK], p-ERK, p38, p-p38, Jun N-terminal kinase 1/2 [JNK1/2], and p-JNK1/2) in lung tissues.

Results: We found that Rosavin significantly attenuated sepsis-induced lung injury. Specifically, Rosavin significantly inhibited inflammation response by decreasing the secretion of inflammatory mediators. The level of neutrophil extracellular traps (NETs) and MPO activity in CLP were decreased after administration with Rosavin. Moreover, the western blot showed that Rosavin could suppress NETs formation by inhibiting the MAPK/ERK/p38/JNK signaling pathway.

Conclusion: These findings demonstrated that Rosavin inhibited NETs formation to attenuate sepsis-induced lung injury, and the inhibitory effect may be exerted via deregulation of the MAPK pathways.

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KEYWORDS
MAPK Pathways; Neutrophil Extracellular Traps; Rosavin; Sepsis-induced Lung Injury

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Rosavin ameliorates sepsis-induced lung injury

Introduction

Sepsis is an infection-induced multiorgan dysfunction disease that is manifested with increased vascular permeability, which can lead to acute respiratory and organ failure, and can even be life threatening. The epidemiological study also demonstrated that the primary and most prevalent cause of mortality was lung tissue dysfunction such as acute respiratory distress syndrome (ARDS). Sepsis-induced dysfunction of lungs includes endothelial damage, excessive inflammatory responses, and an abundance of neutrophil extracellular traps (NETs). Neutrophils are the first barrier against inflammation and the main reaction cells in inflammatory responses. Specifically, NETs are neutrophil reticular extracellular structures consisting of DNA, histones, myeloperoxidase (MPO), and neutrophil elastase. During the localized stage of infection, NETs capture and kill pathogens, which help limit the progression of inflammation. However, during the systemic infection phase of sepsis, NETs increase the morbidity and mortality of sepsis due to their toxicity to lung epithelial cells that leads to acute lung injury.

Traditional Chinese medicine has shown to have potent pharmacological functions in the treatment of lung diseases. Rosavin, an alkyl benzene disaccharide, is one of the most crucial active components of Rhodiola, which has been reported to have antidepressant, anticancerous, and antioxidant effects. Recently, several studies have shown that Rosavin inhibits RANKL-induced osteoclast formation and suppresses inflammatory responses, thereby inhibiting bone loss. Rosavin also inhibits bleomycin induction-induced pulmonary fibrosis and inflammation signaling pathways and alleviates lung injury. Rosavin pretreatment ameliorates PM2.5-related lung damage in mice through anti-ferroptosis of the PI3K/Akt/Nrf2 signaling pathway. However, Rosavin has been less studied in sepsis, and its mechanism is unclear.

It has been suggested that Rosavin may alleviate LPS-related lung injury by reducing the formation of NETs. Other studies have also found that Rosavin also inhibits the phosphorylation of ERK1/2. Thus, our study aimed to investigate whether Rosavin attenuated sepsis-induced lung injury by regulating MAKP/ERK signaling pathways and inhibiting the formation of NETs.

Methods

Animals

Twenty-four male Balb/c mice aged 6–8 weeks were provided by Shanghai Experimental Animal Center (Shanghai, China). The mice were maintained in a sanitary setting for a week before the experiment. This procedure was approved by the Ethics Committee of Hangzhou Cancer Hospital.

Cecal ligation and puncture model

Mice were subjected to the same CLP procedure as before. Briefly, mice were rendered unconscious, and a midline incision was produced. Twenty-two gauge needles were used to ligate and penetrate the cecum. A small amount of excrement was extracted and restored to its usual place, and the incision was closed. The mice in the sham group were treated identically; however, the ligation and puncture processes were neglected. 24 h following the surgery, the bronchoalveolar lavage fluid (BALF) and lung tissues of mice treated with CLP or sham procedure were collected for examination.

Rosavin administration

Rosavin was purchased from Nuodande Standard Technical Services (Rosavin ≥ 98%, China). The mice were randomly separated into four groups: Sham group, CLP group, CLP + 2.5 mg/kg Rosavin, and CLP + 5 mg/kg Rosavin, with each group consisting of six mice. The CLP + Rosavin-treated groups received an intraperitoneal injection of 2.5 mg/kg or 5 mg/kg Rosavin after CLP surgery. The Sham group received an equivalent volume of phosphate-buffered saline (PBS) intraperitoneally.

Lung histological test and lung injury scores

Lung tissues were embedded in paraffin, cut into sections, and fixed with 4% paraformaldehyde. Sections were stained with hematoxylin and eosin, and the images were acquired with a light microscope. The Mikawa technique was used to assess lung damage scores in accordance with the following four 0–4-graded objectives: respiratory congestion, hemorrhage, neutrophils infiltration, and alveolar wall thickening or a transparent film formation. Lung damage was scored on a 5-point scale as follows: 0 = minimal damage, 1+= mild damage, 2+= moderate damage, 3+= severe damage, and 4+= maximal damage.

Lung wet/dry weight ratio

The ratio of wet to dry weight in lung tissue was used to assess pulmonary edema. The lung tissue was separated from the upper left lung lobe. After draining the water, the tissues were weighed twice before dehydrating for 24 h at 80°C. The findings were determined by dividing the wet weight by dry weight.

Enzyme-linked immunosorbent assays (ELISA) assay

The BALF samples of mice were collected and stored at −80°C. The levels of TNF-α, IL-6, IL-1β, and IL-17A were measured using the Invitrogen ELISA kits (Carlsbad, CA, USA).

Flow cytometry

BALF cells were collected from the lung tissues of mice, washed, and stained with anti-mouse Ly6G+ labeled neutrophil according to the instructions. Data analysis was performed using FlowJo software V10.
**Immunofluorescence assay**

To ascertain the MPO-positive cells, immunofluorescence tests were conducted. Lung tissue paraffin sections were deparaffinized with varying doses of dimethylbenzene and ethanol. Antigen was extracted using EDTA retrieval buffer, and sections were blocked for 60 min with 10% donkey serum. The sections were then treated overnight at 4°C with primary antibodies against MPO and histone (1:100, Abcam, Cambridge, UK). After rinsing with PBST, the sections were incubated with secondary antibody (1:1000) at room temperature for 1.5 h. The nucleus was stained for 15 min with 4',6-diamidino-2-phenylindole (DAPI). The sections were observed under a fluorescent microscope in the final step (Leica, Wetzlar, Germany).

**Western blot**

The expressions of p-ERK, ERK, p-p38, p38, p-JNK1/2, JNK1/2, and β-actin in BALF were examined by western blotting. The upper left lung lobe was homogenized, and total proteins were extracted using a tissue protein extraction reagent. The protein concentration was then determined using a bicinchoninic acid kit (BCA; Beyotime, Shanghai, China). Equivalent amounts (10 μg) of denatured proteins were electrophoretically separated based on molecular weight before being transferred to polyvinylidene difluoride membranes (LMAI Bio, LM1136). The membranes were blocked with 5% nonfat dry milk for an hour at room temperature. They were incubated with matching secondary antibodies for an hour at 37°C after being incubated with primary antibodies for an overnight period at 4°C, and rinsed with TBST. The particular protein bands were checked using the enhanced chemiluminescence detection technique (GE Healthcare, Piscataway, NJ, USA).

**Results**

**Rosavin alleviates sepsis-induced lung injury**

Rosavin is now known to have anti-inflammatory effects. To evaluate the possible protective roles of Rosavin in lung injury induced by sepsis, the sepsis mouse model was established by CLP and were administrated with different concentrations of Rosavin (2.5 mg/kg or 5 mg/kg). Lung tissues exhibited extensive histopathological damage, including alveolar wall thickening, interstitial edema, and pulmonary congestion in the CLP group compared with the sham group, while the administration of Rosavin significantly reduced the severity of CLP-induced lung injury in a dose-dependent manner (Figure 1A). Specifically, quantitative analysis of the lung injury score showed that the damage of CLP group was significantly higher than the

![Figure 1](image_url)  
**Figure 1** Rosavin alleviates sepsis-induced lung injury. (A) Lung tissues from each experimental group were processed for histological evaluation at 24 h after CLP injury. Representative images of lung sections stained with H&E staining, the magnification of histological and microscopic images is 200X and 400X, respectively. (B) The lung injury scores were estimated by the Mikawa method. (C) Lung tissue edema was measured by the lung wet/dry weight ratio. Data represent the mean ± SD. **P < 0.001 vs the sham group. *P < 0.05, **P < 0.01 vs the CLP group.
Rosavin reduces the release of sepsis-induced inflammatory factors

Regarding the anti-inflammation effect of Rosavin on sepsis, the contents of inflammatory mediators in BALF, including the TNF-α, IL-6, IL-1β, and IL-17A, were detected by ELISA. The result suggested that CLP induced an increased level of relevant inflammatory mediators compared with the sham group (P < 0.001, Figure 2), while treatments with Rosavin could decrease the levels of TNF-α, IL-6, IL-1β, and IL-17A in BALF compared with the CLP group (P < 0.001, Figure 2).

Rosavin inhibits the formation of neutrophils extracellular trap network

Neutrophils are the main responding cells in the inflammatory response to sepsis, and it has been reported that the anti-inflammatory effect of Rosavin may be related to the inhibition of neutrophil NET formation.4,11,14 The number of neutrophils (Ly6G+ cells) in BALF was measured using flow cytometry, which demonstrated that CLP significantly increased neutrophil cells in BALF compared with that of control, whereas the number of neutrophils was dramatically reduced by Rosavin treatment (P < 0.001, Figures 3A and 3B). In addition, as Rosavin treatment reduced inflammatory reactions and neutrophil overactivation, both of which contributed to the development of NET,22 we then identified the NET-related markers (histone and MPO) in the Rosavin administration group. According to the immunofluorescence analysis, the overlap of MPO and histone was increased in lung tissues of the CLP group, while Rosavin treatment inhibited lung NET levels (P < 0.001, Figure 3C). These findings indicated that CLP induced neutrophil infiltration and the formation of NETs, whereas Rosavin treatment attenuated these effects.

Rosavin inhibits the activation of MAPK pathways

A number of intercellular processes, including growth, development, and differentiation, are mediated by the MAPK-ERK/p38/JNK signaling pathways, and it has been demonstrated that these pathways are crucial in the inflammatory reactions induced by sepsis.23 Therefore, the expressions of p-ERK, ERK, p-p38, p38, p-JNK1/2, and JNK1/2 in lung tissue were assessed by western blot. Results indicated that the relative expressions of p-ERK/ERK, p-p38/p38, and p-JNK1/2/JNK1/2 in the CLP group were increased in comparison to the sham group. After Rosavin treatment, the MAPK pathways-related molecules were significantly decreased (P < 0.001, Figure 4). Furthermore, SB 202190, the MAPK inhibitor, was applied to CLP + Rosavin group. The results indicated that with the application of SB 202190 the effects of Rosavin on CLP-induced lung injury was dramatically enhanced, as manifested by a decrease in lung wet/dry weight ratio, the levels of inflammatory factors, and the number of neutrophils (Figure 5). Together, these results demonstrated that the administration of Rosavin exerts a protective effect on sepsis-induced lung injury by inhibiting MAPK-related pathways.

Discussion

Our study showed that Rosavin attenuated sepsis-induced lung injury. The pulmonary inflammatory response in CLP mice was inhibited, and the excessive activation of neutrophils was inhibited by Rosavin administration. NETs formation in BALF of lung tissues was also greatly reduced. The mechanistic study showed that the inhibitory effect of Rosavin on sepsis-induced lung injury may be exerted via deregulation of the MAPK-ERK/p38/JNK signaling pathways.

Pulmonary injury induced by sepsis is mainly manifested as severe alveolar wall thickening, interstitial edema, and congestion, with an increase in inflammatory reactions. Our study successfully established the sepsis model by CLP, which revealed severe lung histopathological damage on examination of the lung injury score and lung wet/dry weight ratio. The treatment using Rosavin showed amelioration of CLP-related lung injury.

The overactive inflammatory reaction is closely involved in sepsis-induced lung injury. Park et al.24 found that the lung histopathological damage induced by CLP was enhanced in murine by facilitating the release of inflammatory cytokines including TNF-α, IL-6, IL-1β, and IL-17A. In sepsis patients, Fractalkine aggravates sepsis pulmonary damage, and the serum level of Fractalkine positively correlated with the number of neutrophils and the levels of inflammation factors (increased IL-6, IL-1β, IL-17A, IFN-γ, and TNF-α).25 Similarly, our study showed that CLP significantly induced neutrophil overactivation and inflammatory

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**Figure 2.** Rosavin reduces the release of sepsis-induced inflammatory factors. The expression of inflammatory factors in BALF, including TNF-α, IL-6, IL-1β, and IL-17A, were measured by ELISA assays. Data represent the mean ± SD. ***P < 0.001 vs the sham group. **P < 0.001 vs the CLP group.
Figure 3  Rosavin inhibits the formation of neutrophils extracellular trap network. (A) The number of neutrophils (Ly6G+ cells) in BALF was detected using flow cytometry. (B) Histograms show the quantitative of Ly6G+ cells in BALF. (C) Neutrophil extracellular trap amounts were measured by immunofluorescence assay. (D) Histograms show the quantitative of MPO and histone stain in immunofluorescence. Data represent the mean ± SD. ***P < 0.001 vs the sham group. ###P < 0.001 vs the CLP group.

Figure 4  Rosavin inhibits the activation of MAPK pathways. The expression of p-ERK1/2, ERK1/2, p-p38, p38, p-JNK1/2, JNK1/2, and β-actin in lung tissue was detected using western blot. Histograms show the relative gray value of proteins evaluated by Image Lab. Data represent the mean ± SD. ***P < 0.001 vs the sham group. **P < 0.01, ###P < 0.001 vs the CLP group.
reactions compared to the control. However, these effects were dramatically reduced by Rosavin treatment.

Furthermore, NET formation is an important mechanism in sepsis-induced lung damage. Both inflammatory and oxidative reactions stimulate the formation of NETs, and as the main inflammatory response cells, the neutrophils, stimulate intracellular signaling cascade reactions, accelerating the release of NETs. Additionally, neutrophils stimulate massive ROS release, which activates MPO and further promotes the formation of NETs. In this study, Rosavin showed anti-inflammatory effects in CLP-induced sepsis mice. Therefore, we hypothesized that Rosavin may reduce NETs formation. Interestingly, based on the fluorescence intensity of histones and MPO levels in BALF, the number of neutrophils and their MPO levels in BALF were significantly elevated in the lungs of CLP-stimulated sepsis mice, while NETs in the lung tissues were significantly reduced after administration of Rosavin. These findings are in line with the pulmonary histopathology results, indicating that Rosavin alleviated lung damage by inhibiting excessive inflammatory reaction and decreasing the NETs formation.

The MAPK-ERK/p38/JNK signaling pathway has been demonstrated to be crucial in the inflammatory reactions induced by sepsis. Previous studies have found that Rosavin could inhibit MAPK-induced phosphorylation of ERK, p38, and JNK, thereby suppressing osteoclastogenesis and balancing bone homeostasis, which suggested that the anti-inflammatory effect of Rosavin may be exerted by blocking of the MAPK/ERK/p38/JNK signaling pathway. In our study, we examined the levels of p-ERK/ERK, p-p38/p38, and p-JNK/JNK in lung tissue. Fortunately, the results demonstrated that Rosavin treatment significantly reduced the phosphorylation of the MAPK/ERK/p38/JNK pathway, which may contribute to attenuation of sepsis-induced lung injury.

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Competing Interests

The authors state that there are no conflicts of interest to disclose.

Ethical Approval

Ethical approval was obtained from the Ethics Committee of Hangzhou Cancer Hospital.

Consent to Participate Statement

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.
Data Availability

The authors declare that all data supporting the findings of this study are available within the article and any raw data can be obtained from the corresponding author upon request.

Authors’ Contributions

Tianwei Gao and Juan Li designed the study and carried them out. Tianwei Gao, Juan Li, Lei Shi, and Bo Hu supervised the data collection, and analyzed and interpreted the data. Tianwei Gao and Juan Li prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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Figure S1  The chemical structure of Rosavin.