Shenfu Injection Mitigates Sepsis-Induced Acute Lung Injury in Rats by Modulating the RIPK3 Signaling Pathway

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Abstract: Objective: This research explored the protective role and underlying mechanism of Shenfu Injection (SFI) in a rat model of sepsis-induced acute lung injury (ALI), with particular attention to the modulation of the receptor-interacting protein kinase 3 (RIPK3) signaling pathway. Methods: A rat model of sepsis-induced acute lung injury (ALI) was generated through the cecal ligation and puncture (CLP) procedure. Animals were randomly assigned to four groups: Sham, CLP, CLP+SFI (10 mL/kg, intraperitoneal), and CLP+SFI high-dose (20 mL/kg). After 24 hours, lung and serum samples were collected. Histopathological alterations in pulmonary tissues were examined using hematoxylin-eosin (H&E) staining and evaluated by lung injury scoring. The wet-to-dry (W/D) ratio was calculated to assess pulmonary edema. Serum concentrations of TNF-α and IL-6 were quantified by ELISA. Expression levels of RIPK3 and mixed lineage kinase domain-like protein (MLKL) in lung tissues were analyzed through Western blotting and immunohistochemistry. Results: Relative to the CLP group, Shenfu Injection (SFI) treatment notably mitigated alveolar structural impairment, suppressed inflammatory cell infiltration, and lowered lung injury scores. SFI administration also markedly reduced the pulmonary wet-to-dry (W/D) ratio and serum concentrations of TNF-α and IL-6. Western blotting revealed that the CLP group exhibited elevated expression of RIPK3 and phosphorylated MLKL, whereas these proteins were significantly diminished in rats receiving SFI. Conclusion: Shenfu Injection (SFI) alleviates sepsis-induced acute lung injury in rats through the suppression of inflammatory responses and the inhibition of RIPK3/MLKL-dependent necroptotic signaling. These results indicate that regulation of the RIPK3 pathway could represent a key mechanism contributing to the protective action of SFI against sepsis-related organ damage.

Keywords: Sepsis; Acute lung injury; Shenfu Injection; RIPK3; Necroptosis; Inflammation

1. Introduction

Sepsis is a severe, life-threatening disorder marked by an imbalanced host response to infection, leading to multiple organ dysfunction[1]. Acute lung injury (ALI) and its advanced stage, acute respiratory distress syndrome (ARDS), represent some of the most common and deadly complications associated with sepsis[2]. Although critical care strategies have improved, mortality in sepsis-induced ALI remains high, largely owing to the absence of effective pharmacological treatments.

Shenfu Injection (SFI), a modern preparation derived from the classical traditional Chinese medicine formula Shenfu Decoction, is composed of red ginseng (Panax ginseng) and aconite (Aconitum carmichaelii). It has been widely applied in China for managing shock and heart failure, exhibiting beneficial actions such as enhancing circulation, improving cardiac contractility, and preventing mitochondrial damage. Recent research indicates that SFI possesses anti-inflammatory, anti-apoptotic, and immunomodulatory properties in sepsis and critical illness models.

Receptor-interacting protein kinase 3 (RIPK3) functions as a central regulator of programmed necrosis (necroptosis), a controlled cell death pathway that contributes significantly to

inflammation and organ damage during sepsis[3]. Upon activation, RIPK3 and its downstream target, mixed lineage kinase domain-like protein (MLKL), trigger plasma membrane disruption and the release of pro-inflammatory mediators, exacerbating tissue injury[4–5]. Consequently, inhibition of RIPK3-dependent necroptosis has been proposed as a promising therapeutic strategy for mitigating sepsis-induced organ dysfunction[6].

The present research was designed to investigate the protective role of Shenfu Injection in a rat model of sepsis-induced ALI and to elucidate its potential mechanism through regulation of the RIPK3 signaling pathway.

2. Experimental Design and Methods

2.1 Laboratory Animals

Male Sprague-Dawley rats weighing 220–250 g were supplied by the Experimental Animal Center of Nanchang Medical College. The animals were maintained under standardized environmental conditions (temperature 22 ± 2 °C, 12 h light/dark cycle) and provided with unrestricted access to standard chow and water. All procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Jiangxi Provincial People's Hospital.

2.2 Experimental groups and treatments

The rats were randomly divided into four groups, with ten animals in each group:

1) Sham group: rats underwent a laparotomy procedure without performing cecal ligation or puncture;

2) CLP group: cecal ligation followed by puncture to establish a sepsis model;

3) CLP + SFI (Low dose): CLP rats treated with Shenfu Injection 10 mL/kg intraperitoneally;

4) CLP + SFI (High dose): CLP rats treated with Shenfu Injection 20 mL/kg intraperitoneally.

SFI (Ya'an Sanjiu Pharmaceutical Co., Ltd., China) was administered immediately after CLP and repeated every 12 h for 24 h.

2.3 Modeling Sepsis-Induced Acute Lung Injury in Rats

The cecal ligation and puncture (CLP) operation was carried out according to established protocols. Following anesthesia with intraperitoneal pentobarbital sodium (50 mg/kg), a midline abdominal incision was made to expose the cecum, which was ligated at approximately half its length and punctured twice using an 18-gauge needle. A small quantity of fecal matter was gently extruded before returning the cecum to the peritoneal cavity. The abdominal wall was then closed, and 2 mL of sterile saline was administered subcutaneously for fluid resuscitation. Rats in the sham group underwent identical surgical manipulation except for ligation and puncture.

2.4 Histopathology and Lung Injury Scoring

After 24 hours, lung samples were preserved in 4% paraformaldehyde, paraffin-embedded, and sliced into 5-μm sections. The tissue sections were then stained with hematoxylin and eosin (H&E) and examined using a light microscope. Pulmonary injury was assessed according to the degree of alveolar edema, inflammatory infiltration, hemorrhage, and thickening of the alveolar septa, with a grading scale from 0 (normal) to 4 (severe).

2.5 Assessment of Lung Water Content (W/D Ratio)

The upper right lung lobe was weighed immediately after excision to determine the wet weight, followed by drying at 60 °C for 72 hours to obtain the dry weight. The wet-to-dry (W/D) ratio was subsequently computed as an indicator of pulmonary edema.

2.6 ELISA for Cytokines

Levels of TNF-α and IL-6 in serum were quantified with rat-specific ELISA kits (Elabscience, Wuhan, China) following the manufacturer's protocol.

2.7 Protein Expression Analysis by Western Blotting

Total proteins were isolated from lung tissues with RIPA lysis buffer. Equivalent protein quantities were resolved by SDS-PAGE and subsequently transferred onto PVDF membranes. After blocking, the membranes were incubated overnight at 4 °C with primary antibodies against RIPK3, phosphorylated MLKL (p-MLKL), total MLKL, and β-actin, followed by incubation with HRP-linked secondary antibodies. Protein bands were detected using an enhanced chemiluminescence (ECL) system, and relative expression levels were analyzed through densitometric evaluation.

2.8 Immunohistochemistry

Paraffin-embedded lung sections were deparaffinized, rehydrated, and processed for antigen retrieval. Following blocking, the sections were incubated overnight with an anti-RIPK3 primary antibody, then treated with a corresponding secondary antibody and visualized using DAB chromogenic reagent. The staining intensity of RIPK3 was assessed semi-quantitatively under a light microscope at 400× magnification.

2.9 Statistical Evaluation

All data are presented as the mean ± standard deviation (SD). Statistical analysis was conducted using one-way ANOVA with Tukey's post hoc test for multiple comparisons. Differences were regarded as statistically significant when $p < 0.05$.

3. Research Outcomes

3.1 Shenfu Injection Attenuated Lung Histopathological Damage

Hematoxylin and eosin (H&E) staining revealed intact and well-preserved alveolar structures in the Sham group. In the CLP group, alveolar structures were severely destroyed, with extensive edema, hemorrhage, and neutrophil infiltration. Treatment with Shenfu Injection significantly ameliorated these pathological changes in a dose-dependent manner. The SFI-treated groups exhibited significantly reduced lung injury scores relative to the CLP group (**Figure 1.**).

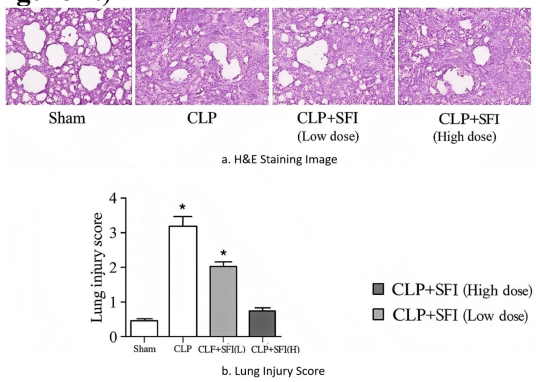

Figure 1. Histopathological Analysis (H&E Staining)

3.2 SFI Reduced Lung Edema

The wet-to-dry (W/D) lung weight ratio, reflecting lung water accumulation, was markedly increased in the CLP group relative to the Sham group ($p < 0.01$). Administration of SFI at both low and high doses significantly reduced the W/D ratio ($p < 0.05$ and $p < 0.01$, respectively), suggesting enhanced clearance of alveolar fluid (**Figure 2.**).

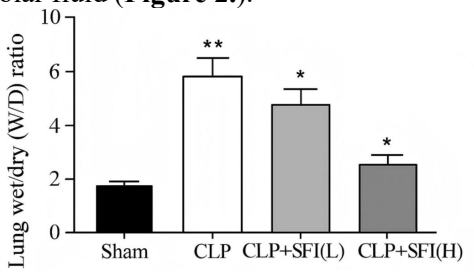

Figure 2. Lung Wet/dry (W/D) Ratio

3.3 SFI Inhibited Systemic Inflammation

CLP induction led to a marked elevation in serum TNF-α and IL-6 concentrations ($p < 0.01$ vs. Sham). Treatment with Shenfu Injection (SFI) markedly decreased the levels of both inflammatory cytokines, with a more pronounced reduction observed in the high-dose group (**Figure 3.**).

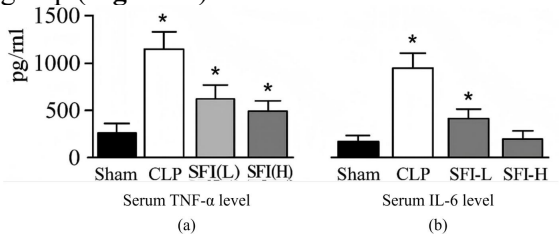

Figure 3. Serum TNF-α and IL-6 Levels

3.4 SFI suppressed RIPK3/MLKL signaling activation

Western blot analysis revealed a significant increase in RIPK3 and phosphorylated MLKL (p-MLKL) expression in the CLP group. Administration of Shenfu Injection (SFI) notably suppressed the expression of RIPK3 and p-MLKL, whereas total MLKL levels showed no evident alteration. Immunohistochemical staining further confirmed that SFI reduced RIPK3-positive cell numbers in lung tissue (**Figure 4.**).

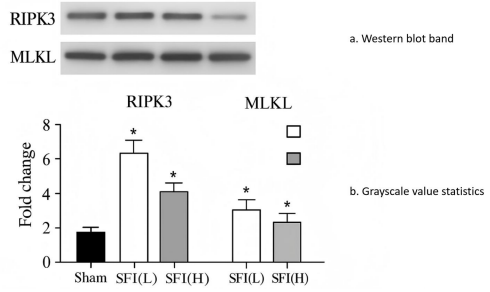

Figure 4. RIPK3 and MLKL Protein Expression

3.5 Immunohistochemical Detection of RIPK3 in Lung Tissue

In the Sham group, only weak background staining was observed, indicating low basal RIPK3 expression in normal lung tissue. In contrast, the CLP group exhibited markedly increased brown DAB-positive staining, predominantly distributed in alveolar epithelial cells and inflammatory infiltrating cells, suggesting a strong activation of RIPK3 in septic lung injury. Both SFI-treated groups showed visibly reduced RIPK3 staining intensity compared with the CLP group, with the high-dose SFI group displaying a more pronounced decrease. The semi-quantitative analysis of integrated optical density (IOD) confirmed the visual observation: RIPK3 expression was markedly increased in the CLP group ($p < 0.01$ vs. Sham). Treatment with Shenfu Injection (SFI) significantly attenuated RIPK3 levels in a dose-dependent fashion ($p < 0.05$ for the low-dose and $p < 0.01$ for the high-dose group). These findings (**Figure 5.**) support the hypothesis that SFI alleviates septic lung injury at least in part by suppressing RIPK3-mediated necroptotic signaling at the tissue level.

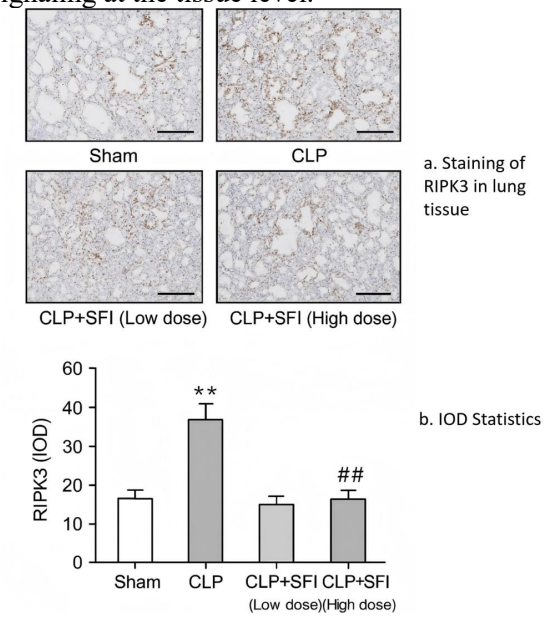

Figure 5. Immunohistochemistry (IHC) Result Image

4. Discussion

In this study, Shenfu Injection (SFI) attenuated CLP-induced acute lung injury (ALI) by reducing inflammatory cytokine release, decreasing pulmonary edema, improving histological architecture, and suppressing activation of the RIPK3/MLKL necroptotic pathway. These data indicate that modulation of regulated necrosis may be a key mechanism by which SFI confers organ protection in sepsis[7].

Necroptosis, a programmed form of cell death predominantly governed by the RIPK1–RIPK3–MLKL signaling axis, has been recognized as a major mechanism contributing to inflammation-associated tissue injury in sepsis and various critical conditions[8]. Upon activation, RIPK3 phosphorylates MLKL, leading to its oligomerization and translocation to the plasma membrane, where it disrupts membrane integrity and promotes the release of damage-associated molecular patterns (DAMPs). This cascade both executes cell death and amplifies inflammatory signaling, creating a feed-forward loop that exacerbates organ dysfunction[9]. Recent mechanistic investigations and review articles have highlighted the pivotal involvement of RIPK3 in sepsis-associated tissue injury and immune imbalance, underscoring necroptosis as a potential therapeutic target for sepsis[10].

Our observation that CLP increased RIPK3 and p-MLKL levels in lung tissue, and that SFI suppressed both, aligns with the concept that inhibiting necroptotic signaling can mitigate lung injury[11]. Mechanistically, SFI contains bioactive constituents derived from Panax ginseng (ginsenosides) and Aconitum species (aconite alkaloids), which have been reported to possess anti-inflammatory, antioxidant, and mitochondrial-stabilizing activities[12]. Preclinical studies of individual ginsenosides (e.g., Rb1, Rg1) indicate modulation of NF-κB, MAPK and mitochondrial pathways, leading to reduced cytokine production and improved cellular survival in sepsis models; such pleiotropic effects could indirectly reduce triggers of necroptosis (e.g., TNF family signaling, oxidative stress), thereby lowering RIPK3 activation[13].

In addition to indirect anti-inflammatory effects, our results raise the possibility that SFI or its constituents may directly interfere with components of the necroptotic machinery or their upstream regulators[14]. While small-molecule RIPK3 inhibitors and experimental agents that block necroptosis have shown organ-protective effects in animal models, translating these inhibitors clinically has been challenging and remains an active area of drug discovery. The observed downregulation of RIPK3/p-MLKL by SFI suggests that botanical preparations warrant investigation as alternative

or adjunctive modulators of regulated necrosis, whether by transcriptional regulation, inhibition of upstream kinase activation, post-translational modification of necrosome components, or enhancement of cellular clearance mechanisms[15].

Beyond necroptosis, recent literature reveals intricate crosstalk among regulated cell death pathways (necroptosis, apoptosis, pyroptosis, ferroptosis) and innate immune sensors (for example, NLRP3 inflammasome and cGAS-STING), which together determine the magnitude and phenotype of sepsis-induced inflammation. RIPK3 has been reported to promote NLRP3 activation in certain contexts, thereby linking necroptotic signaling to amplified cytokine release (e.g., IL-1β) and inflammasome-dependent injury. Thus, suppression of RIPK3 by SFI could exert broader immunomodulatory effects beyond blockade of membrane rupture alone. Future experiments should measure inflammasome activation, IL-1β release, and markers of other regulated death pathways to map SFI's spectrum of action[16].

Several translational considerations emerge from our findings. Clinically, Shenfu Injection has been used as adjunctive therapy in septic patients in China, and recent clinical and meta-analytic reports suggest potential benefits in selected endpoints such as hemodynamic stability and organ perfusion; however, high-quality randomized trials with hard outcomes (mortality, ventilator-free days) remain limited[17-18]. Our preclinical demonstration that SFI modulates RIPK3/MLKL signaling provides a mechanistic rationale to include biomarkers of necroptosis (e.g., circulating RIPK3, p-MLKL fragments, DAMPs) in future clinical investigations to identify responders and to translate mechanism-driven dosing strategies[19-20].

Several limitations of this study should be recognized. Firstly, the present work used only two SFI doses and assessed outcomes at a single early time point (24 h); longer time courses and dose-response investigations are necessary to define the therapeutic window and durability of the protective effect. Second, although we measured key necroptotic proteins (RIPK3, p-MLKL), we did not employ genetic (e.g., RIPK3-/- animals) or pharmacologic RIPK3 blockade controls to prove causal dependence; such loss-of-function approaches would strengthen causal inference. Third, SFI is a multicomponent preparation; identification of the active constituent(s) responsible for necroptosis modulation (and their direct molecular targets) remains an important next step. Finally, sepsis is heterogeneous pathogen type, timing, and host factors influence necroptosis contributions, so validating findings across different sepsis models (e.g., LPS, bacterial pneumonia) and species will increase translational robustness.

In conclusion, our data demonstrate that Shenfu Injection ameliorates sepsis-induced ALI in rats and suppresses RIPK3/MLKL necroptotic signaling, supporting a dual anti-inflammatory and anti-necroptotic mechanism. These findings justify more detailed mechanistic studies (including genetic/pharmacologic RIPK3 manipulation), isolation of active constituents, and translational trials incorporating necroptosis biomarkers to evaluate whether SFI-based strategies can be integrated into evidence-based sepsis care.

5. Conclusion

Shenfu Injection (SFI) markedly alleviates septic lung injury in rats through suppression of inflammatory responses, mitigation of pulmonary edema, and inhibition of the RIPK3/MLKL-mediated necroptotic signaling pathway. These results offer experimental support for the potential clinical use of SFI as an adjuvant treatment for sepsis-related lung injury.

Acknowledgments

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