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**Research Article**

# Protective Mechanism of Tong Fu Xie Fei on Lung Barrier Function in Acute Lung Injury Mouse Model

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## Abstract
**Objective:** To explore the protective mechanism of Tong Fu Xie Fei granule on lung barrier function in acute lung injury（ALI）of mice**. Methods:** Mice were randomly assigned to the following four groups, the control group, the model group, the treatment group and the SP group. Each group comprised of 15 mice. Lipopolysaccharide was injected intraperitoneally to induce ALI. Survival rates were recorded for 7 days after modeling. Expression of TNF-α and IL-6 in lung tissues and bronchoalveolar lavage fluid were measured using ELISA. mRNA and protein levels of ZO-1, VE-Cadherin and MLCK were ascertained by qRT-PCR or Western blotting. Optical microscopy was used to examine lung tissue for pathological changes. **Results:** Compared with the control group, there was obvious inflammatory response in the model group and SP group, the treatment group showed moderate inflammatory response and ameliorative lung barrier function than model group and SP group. Significant decreases in the expression levels of ZO-1 and VE-Cadherin, but increases in levels of MLCK and MLC were observed in model and excited group comparing with the control group. The expression levels of ZO-1 and VE-Cadherin were increased, but levels of MLCK and MLC were decreased significantly in the treatment group comparing with the model group and excited group. **Conclusions:** Tong Fu Xie Fei can reduce acute lung injury through effectively alleviating pulmonary inflammation and the activity of MLCK pathway, and enhancing the protective function of lung barrier.

**Key words:** Tongfuxiefei Formula; Acute Lung Injury (ALI); Endothelial barrier function; Myosin Light Chain Kinase (MLCK).

## Introduction

Acute Lung Injury (ALI) is a common clinical critical disease. The main factors causing ALI include systemic infection, sepsis, trauma, shock and poisoning. The main pathological causes were damage of pulmonary vascular endothelial cells, destruction of endothelial integrity, alveolar-capillary barrier dysfunction, increased vascular permeability, inflammatory cell infiltration and pulmonary edema. If these were not treated promptly, the illness progresses to the Acute Respiratory Distress Syndrome (ARDS), with a fatality rate of up to 40%. The barrier function of endothelial cells is mainly regulated by Myosin Light Chain Kinase (MLCK). MLCK is a serine/threonine specific protein kinase dependent on Calmodulin (CaM), and is encoded by MLCK gene. The genes mlck1, mlck2 and mlck3 can encode different subtypes of MLCK, while the endothelial cells mainly express the mlck1 encoded MLCK, with the molecular weight of about 210 KD. At physiological condition, MLCK is activated by upstream signals, such as Ca2-CaM, and then mediates phosphorylation at Thr18 and Ser19 of Myosin Light Chain (MLC). MLC activates ATP in the head of myosin heavy chain, producing energy for actomyosin restructuring, leading to cell shrinkage, intercellular space formation and cell permeability（1 -2）. In the inflammatory condition, inflammatory mediators, such as activated neutrophils, thromboplastin, histamine, tumor necrosis factor-**α**., through a variety of signaling molecules, such as Ca2+, protein kinase C, Src kinase, nitric oxide synthase, cause endothelial barrier dysfunction. Therefore, multiple signaling pathways and molecules related to MLCK can be used as targets for the prevention and treatment to organ edema and multi-organ dysfunction.

Tong Fu Xie Fei formula is originated from ancient books of Chinese medicine. Some clinical studies of traditional Chinese medicine (TCM) have found that Tong Fu Xie Fei formula can significantly improve lung oxygenation function and barrier function in patients with ALI or ARDS.（3-4）. However, the mechanism of the protective effect of Tong Fu Xie Fei formula to ALI patients is still unclear. Therefore, this study used Tongfuxiefei to treat ALI mice, and explored the protective effect of this formula on lung barrier function of mice and the potential mechanism.

## Methods

### Mice

SPF female BALB/c mice from the experimental animal center of Chongqing medical university were used at 6-7 weeks old, weighing about 25 g, and randomly divided into 4 groups with 10 mice in each group.

### Drugs

Materials and drugs used include lipopolysaccharide (LPS), formamide (Sigma), Substance P (SP, US.Enzo), haematoxylin-eosin (HE), erythrocyte lysate, reverse transcription kit (Takara), SYBR (Toyobo), Trizol, tissue protein extraction reagent, allergic ECL chemiluminescence reagent box (American Thermo Fisher), rabbit anti ZO-1, rabbit anti VE-Cadherin, rabbit anti-MLCK, rat anti-beta actin (Abcam), and MLC antibody kit (CST). Components of Tong Fu Xie Fei formula were rhubarb, glauber's salt, honeysuckle, dandelion, houttuynia, magnolia, immature bitter orange, peach kernel, scutellaria, and liquorice.

### Pathological observation and scoring.

After the establishment of ALI model for 16 h, mice were euthanized. Then lung tissues were taken and fixed with 4% paraformaldehyde and underwent HE staining. The lung histopathological changes of the mice were observed under optical microscope and lung injury score was calculated （5）.

### Bronchoalveolar Lavage Fluid (BALF) collection for examination of the TNF-α and IL-6 levels

0.1 mL of 10% chloral hydrate anesthesia was

injected intraperitoneally in mice, bilateral lung lavage was carried out using 1 mL of 4 ℃ saline for 3 times and BALF was collected. Cells were precipitated and re-suspended in 0.5 mL RBC pyrolysis liquid, reacted for 15 min at room temperature, spun down at 4 ℃ at 2500 r/min for 10 minutes, and counted after re-suspending.

**Exam the TNF-α and IL-6 level in Lung tissue permeability**

After 16 h of modeling, 1% Evans Blue (25 mg/kg body weight) was injected into the tail vein of mice. Two hours later, the mice were sacrificed by carotid artery bleeding, and 10 mL PBS was injected into the right ventricle to perfuse pulmonary vessels until the entire lung became white. After wiping the surface moisture and weighing the lung wet weight, lower lobe was fixed with formamide at 1 mL / 100 mg of lung wet weight, 60 ℃ for 24 h. After reaction and spinning for 30 min, Evans Blue was detected at 620 nm absorbance according to the instruction.

**Statistical**

GraphPad Prism 5.0 software was used to calculate the differences of each group. The measurement data were expressed as mean and standard deviation.

## Result

**Survival and histopathological analysis**

The 7 days survival rate of mice in each group was observed. The 7 days survival rate of mice in the blank group and the control group was 100%. All mice in LPS group and SP group died within 48 h after modeling. The 7 days survival rate of LPS+TFXF group was 20%, significantly higher than that of LPS group and SP group（Fig1A）. The above results showed that Tong Fu Xie Fei formula had a good protective effect on ALI group. When the MLCK signaling pathway was activated, the protective effect of Tong Fu Xie Fei on mice was weakened.

Lung histopathological changes were examined and injury scores were measured. Alveolar structure was destroyed, alveolar wall was thickened, alveolar interstitial edema and a large number of inflammatory cell infiltrations were observed in the LPS group and the SP group, and the injury score was significantly increased compared with the control group. The alveolar structure of LPS+TFXF group was slightly damaged, and the inflammatory reaction was mild (Fig 1B、Fig 1C). The injury score was significantly reduced compared with that of LPS group and SP group. The above results suggest that Tong Fu Xie Fei formula can reduce pulmonary inflammatory reaction and edema in ALI group. When the MLCK pathway is activated, the effect of Tong Fu Xie Fei on reducing pulmonary inflammation and edema is weakened. These results suggest that Tong Fu Xie Fei formula may play a role by inhibiting the MLCK signaling pathway.

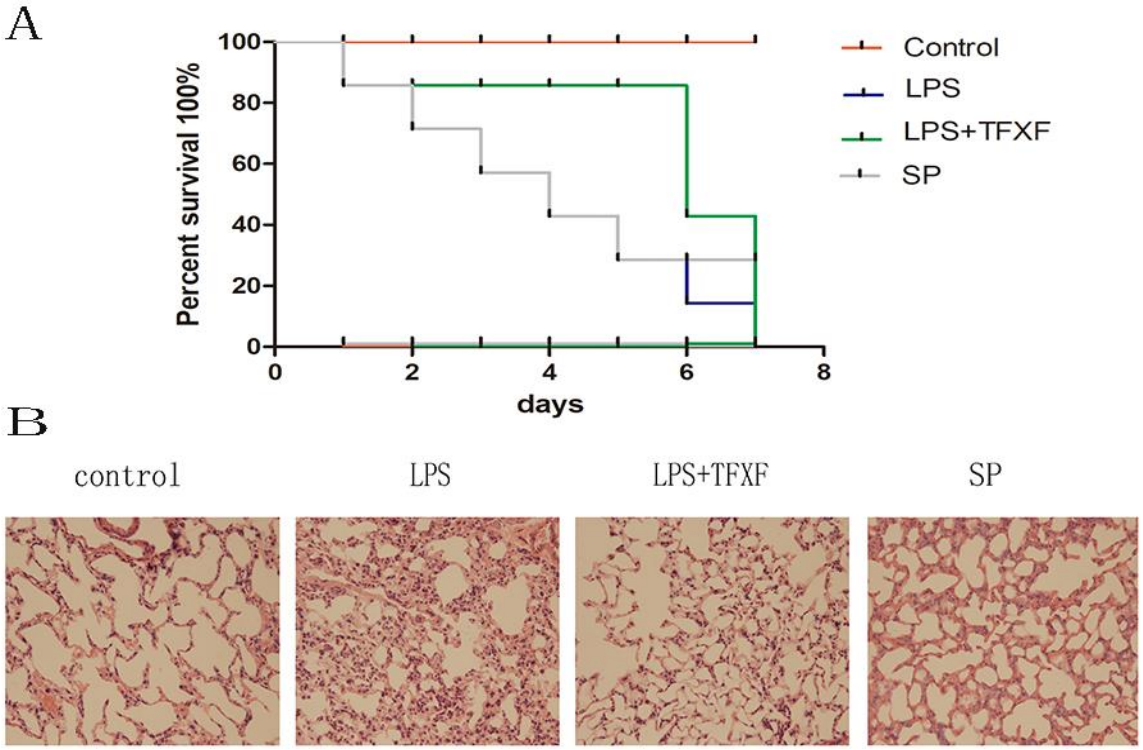
**Lung permeability**

In order to study whether the protective effect of Tong Fu Xie Fei formula on ALI group was based on the effect on the function of pulmonary endothelial barrier, relevant indicators of lung permeability in mice were tested. The lung TNF-α and IL-6 levels of the mice in each group were first detected（Fig 2A）. The TNF-α and IL-6 levels of the LPS group were significantly higher than that of the control group and blank group. The TNF-α and IL-6 levels of lung tissue in LPS+TFXF group were significantly lower than that in LPS group and SP group. The concentration of total protein in BALF of mice was measured. The total protein level in BALF of LPS group was significantly higher than that of blank group and control group (Fig 2C). Histone levels of LPS+TFXF were significantly lower than those of LPS group and SP group. Total levels of TNF-α and IL-6 in BALF LPS group were significantly higher than that of blank group and the control group (Fig 2d); But that in LPS+TFXF group was significantly lower than that in LPS group and SP group. The above results suggested that the lung permeability of acute lung injury mice

was significantly increased compared with the blank group and the control group, while the Tong Fu Xie Fei formula could significantly reduce the lung permeability of mice and improve the lung barrier function of mice. When the agonist SP of MLCK was used to activate this signaling pathway, the protective effect of the formula on the lung barrier was decreased, suggesting that Tong Fu Xie Fei formula may rely on the MLCK signaling pathway to play an important role.

**Expression levels of zo-1, ve-cadherin and MLCK in lung tissues of mice**

In order to further explore the molecular mechanisms of lung tissue permeability, first of all we used qRT - PCR and WB to examine the mRNAs and proteins in pulmonary vascular endothelial protein and adhesion connection protein VE ZO – 1, VE - Cadherin mRNA and protein levels in the lung tissue of LPS mice were decreased significantly than those in the control group (Fig 3A, B, C). The mRNA levels and protein levels of zo-1 and ve-cadherin in the LPS+TFXF group were significantly higher than those in the LPS group, when Tong Fu Xie Fei formula were added, suggesting that this formula could enhance the cellular connectivity on the effect of LPS. When the agonist SP was added, the levels of zo-1 and ve-cadherin in SP group were significantly lower than those in LPS+TFXF group, suggesting that the regulation of zo-1 and ve-cadherin in Tong Fu Xie Fei formula is dependent on MLCK signaling pathway (Fig 3d). To further understand the effect of Tong Fu Xie Fei formula on the MLCK signaling pathway, the level of MLC was detected. The levels of MLCK and MLC in the LPS group were significantly up-regulated compared with the blank group and the control group. The levels of MLCK and MLC were significantly down-regulated compared with those of LPS group under the effect of Tong Fu Xie Fei formula. When SP was added, the inhibitory effect of Tong Fu Xie Fei formula on MLCK and MLC was weakened. The above results suggest that Tong Fu Xie Fei formula may inhibit the MLC by down-regulating the expression level of MLCK, so as to maintain the adhesion connection between endothelial cells and enhance the barrier function of endothelial cells.



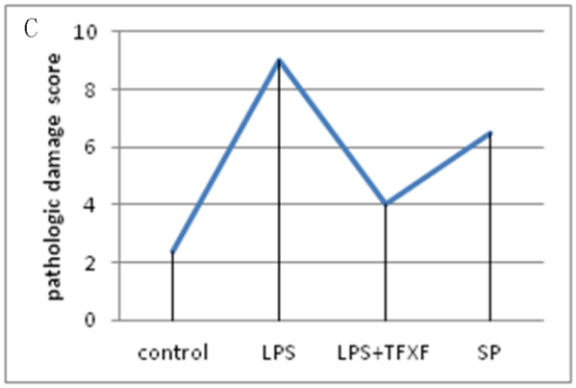



**Fig1. Survival rate (A), lung histopathological changes (B) and injury score (C) of mice.**

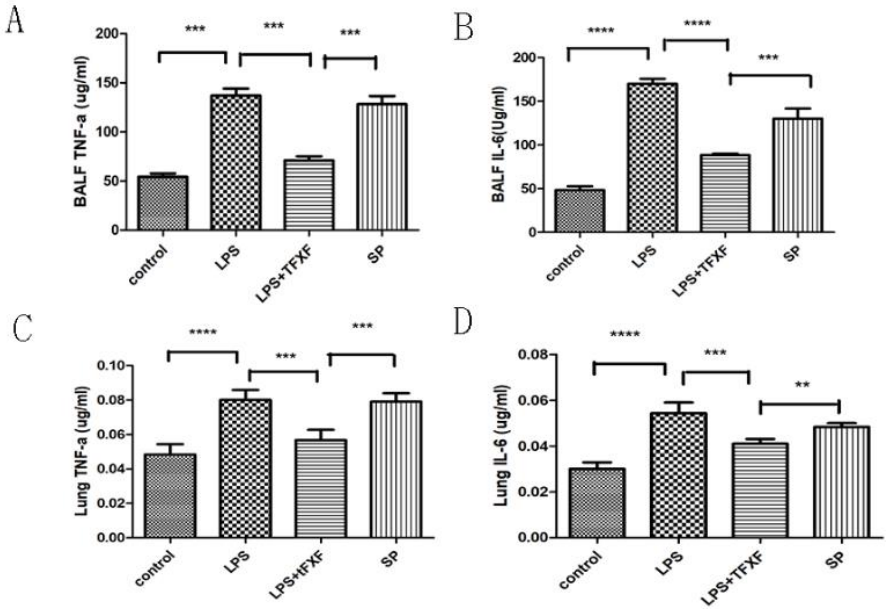



**Fig 2. Changes of relevant indicators of lung tissue permeability in mice.**

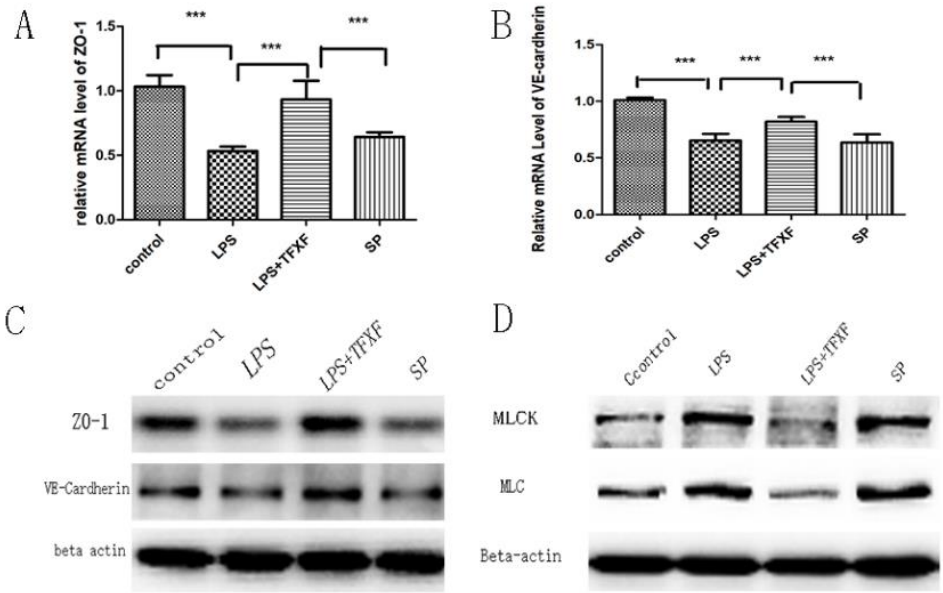



**Fig 3. Expression levels of connective proteins and MLCK pathway molecules in mouse lung tissue.**

## Conclusion

In recent years, some researches have been increasingly conducted on the damage of vascular endothelial barrier caused by acute lung injury. According to literature reports, as some signaling pathways to maintain endothelial integrity, the increased activity of MLCK pathway will cause endothelial barrier dysfunction, leading to increase of the endothelial permeability and inflammation. It is commonly seen in respiratory diseases, cardiovascular diseases, tumors and enteritis inflammatory (6). In some studies on pneumonia, asthma, lung injury, lung cancer and other diseases, the expression of MLCK have been increased (7-8). Some literatures have reported that its epigenetic regulation is closely related to the risk of ARDS in patients (9). Bogatcheva found that LPS influence on human lung microvascular endothelial cells, up-regulating the expression of MLCK, leading to the increase of MLC level, and damaging of the barrier function of endothelial cells (10). Compared with wild-type mice, MLCK knockout mice's endothelial cells were not sensitive to lipopolysaccharide, and could better maintain pulmonary ventilation function, reduce the occurrence of acidosis and pulmonary edema, and ultimately increase the survival rate of mice (11-12). The incidence of acute lung injury of the transgenic mice with MLCK overexpression in endothelial cells was significantly higher than that of wild-type mice, and the survival rate was also significantly reduced (13). These studies provide theoretical support and experimental basis for MLCK as a potential target for the treatment of pulmonary vascular endothelial cell barrier function. Currently, several studies have targeted the inhibition of lung endothelial cells MLCK as one of the strategies for the treatment of ALI. MLCK inhibitors, such as ML-7 and ML-9, can significantly improve pulmonary capillary permeability, reduce pulmonary edema and leukocyte infiltration, and improve lung barrier protection function (14). However, there is no effective Chinese traditional medicine therapy to improve this mechanism. In this study, we used LPS to establish the model of acute lung injury in mice and treat with Tong Fu Xie Fei prescription, which could significantly improve the lung barrier function, reduce pulmonary edema and improve the survival rate of ALI mice by inhibiting the activity of excessive MLCK pathway. This study suggested that the TCM of Tong Fu Xie Fei had certain clinical value in the treatment of acute lung injury induced by LPS.

Many Chinese ancient books have proved the relationship between the lung and large intestine. Based on this theory, this experiment studies the mechanism of Tong Fu Xie Fei and lung barrier function in acute lung injury, which is closely related to the viewpoint of "lung intestinal surface and interior" in TCM theory. Some basic studies have found that Tong Fu Xie Fei can improve hypoxemia and metabolic acidosis in lung injury animals and reduce pulmonary edema by regulating activity of p38 pathway (15) and NLPR3 inflammatory body synthesis (16) antioxidant, inhibiting the expression and release of inflammatory factors, and inhibiting the degradation of pulmonary surfactants (17). The above studies show that Tong Fu Xie Fei can regulate the large intestine clearance function, maintain good water exchange metabolism, detoxify and cool blood, venation and circulation of lung and lung qi, and a series of mechanisms, finally to reduce the lung injury caused by infection, improve the barrier function of lung endothelial cells, reduce inflammation and play the protection role of lung barrier. In this study, it was found for the first time that Tong Fu Xie Fei could regulate the MLCK pathway to improve lung barrier function and reduce pulmonary edema. It is hoped that the results of this study can give theoretical support for Tong Fu Xie Fei prescription in the treatment of ALI from a new angle.

## Declarations

**1)** ***Consent to publication***

We declare that all authors agreed to publish the manuscript at this journal based on the signed Copyright Transfer Agreement, and followed publication ethics.

**2)** ***Ethical approval and consent to participants***

Not applicable.

**3)** ***Disclosure of conflict of interests***

We declare that no conflict of interest exists.

**4)** ***Funding***

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**5)** ***Availability of data and material***

We declare that the data supporting the results reported in the article are available in the published article.

**6)** ***Authors' Contributions***

Authors contributed to this paper with the design (Xi Zhong), literature search (Xi Zhong, Xia Feng, Wen-Hong Peng, Si-Yue Wang and Gang Wang), drafting (Xi Zhong), revision (Xi Zhong and Xia Feng ), editing (Xi Zhong) and final approval (Xi Zhong).

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**8)** ***Authors' biography***

Xi Zhong, born in 1984, is a Ph.D. candidate, and engaged in basic research on traditional Chinese medicine and Western medicine in respiratory critical care medicine.

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