Original Article
Effect of Shenfu injection on lung injury after intestinal ischemia/reperfusion in rats

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Abstract: Growing evidence has highlighted the contribution of gastrointestinal ischemia/reperfusion (IR) in the process of acute lung injury. This study aims to investigate effect of *Shenfu* injection on preventing and treating lung injury caused by intestinal ischemia/reperfusion. Effect of *Shenfu* injection on levels of tumor necrosis factor-α, inducible nitric oxide synthase and intercellular adhesion molecule-1 was also determined. An intestinal ischemia/reperfusion model was established. Sprague-Dawley rats were randomly divided into ischemia/reperfusion, normal control, and *Shenfu* groups. Blood gases and blood lactate, lung wet/dry weight ratio, and myeloperoxidase activity were detected. Mean arterial pressure was monitored before and after reperfusion. The amount of tumor necrosis factor-α in plasma and lung tissue was determined using enzyme-linked immunosorbent assay. Inducible nitric oxide synthase and intercellular adhesion molecule-1 expression in lung and intestinal tissue were detected by immunohistochemistry. *Shenfu* injection significantly attenuated pathological damage in lung caused by intestinal ischemia/reperfusion, improved oxygenation in lungs, and reduced lung wet/dry weight ratio and myeloperoxidase activity. After *Shenfu* injection, tumor necrosis factor-α contents in plasma and lung tissue, and expressions of inducible nitric oxide synthase and intercellular adhesion molecule-1 caused by intestinal ischemia/reperfusion, were inhibited. The mean arterial pressure in ischemia/reperfusion group after reperfusion was significantly decreased compared to *Shenfu* group. In conclusion, *Shenfu* injection could prevent occurrence of lung injury caused by intestinal ischemia/reperfusion and accordingly prevented development of multiple organ dys-function. This effect is achieved through inhibiting release of tumor necrosis factor-α, inducible nitric oxide synthase and intercellular adhesion molecule-1.

Keywords: *Shenfu*, intestinal ischemia/reperfusion, lung injury, tumor necrosis factor-α, inducible nitric oxide synthase, intercellular adhesion molecule-1
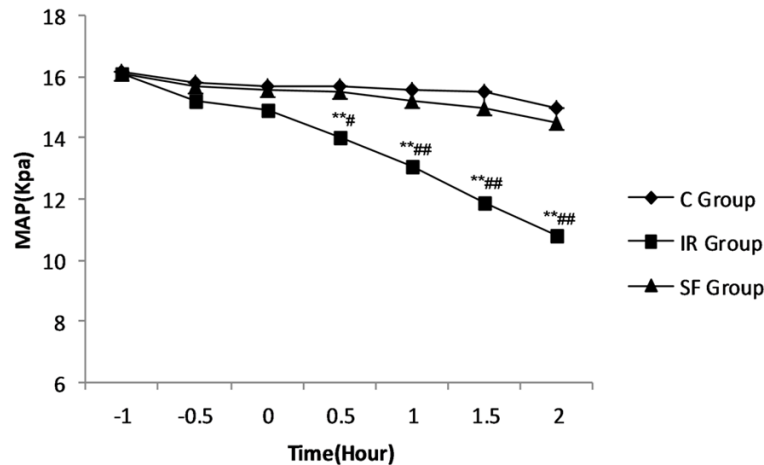
Introduction

Acute lung injury, including acute respiratory distress syndrome and multiple organ dysfunction syndrome, has a 40% mortality rate and is considered a major challenge for physicians working in the intensive care unit [1]. Growing evidence has highlighted the contribution of gastrointestinal ischemia/reperfusion (IR) in this process. Intestinal IR is a pathological process involving multiple factors, which quickly activate local or remote organ and systemic inflammatory responses. These trigger damage to the intestinal tract and remote organs such as the lung, liver, heart and kidney, thus leading to the occurrence of multiple organ dysfunction syndrome. Inflammatory mediators and cyto-

kines play a key role in this pathological process [2, 3]. Tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS) are factors contributing to multiple organ dysfunction syndrome induced by the intestinal IR-caused remote organ damage. The involvement of intercellular adhesion molecule-1 (ICAM-1) has also been attracting increasing attention in recent years [4-6].

Natural chemical libraries and multi-target effects seen with Chinese herbs, including *Shenfu,* have many advantages for use as preventive treatments of IR injury [7]. *Shenfu* injection exerts a variety of pharmacological effects. These effects include the scavenging of oxygen free radicals, regulation of calcium homeosta-

Figure 1. Changes in mean arterial pressure. *P < 0.05, **P < 0.01, vs. control group; #P < 0.05, ##P < 0.01, vs. Shenfu group.

sis, inhibition of the production or release of inflammatory factors, improvement of tissue perfusion and oxygenation, inhibition of lipid peroxidation during IR, and amelioration of multiple organ damage [8-11]. This study aims to explore the protective effect of Shenfu injection on intestinal IR-caused lung injury through observing TNF-α, ICAM-1 and iNOS during the injury process, in a broader attempt to investigate the mechanism associated with Shenfu protection against lung injury.

Materials and methods

Establishment of models and grouping

Healthy male Sprague-Dawley rats were provided by the Experimental Animal Center of Wuhan University (Wuhan, Hubei Province, China). All rats were fasted for 12 hours prior to experimentation, and allowed free access to water. After rats were anesthetized by intraperitoneal injection of 20% urethane 1 g/kg, the left femoral artery was catheterized under sterile conditions and then connected to a polygraph (LIFESCOPE9, Nihon Kohden, Tokyo, Japan) for continuous monitoring of arterial blood pressure and blood sampling. A catheter was also placed in the left femoral vein for injections. A median abdominal incision was performed, and the superior mesenteric artery was separated and occluded using a noninvasive vascular clamp for 1 hour, followed by 2 hours of reperfusion. Thirty-six Sprague-Dawley rats were randomly divided into the IR, normal control, and Shenfu groups, with 12 rats in

each group. In the IR group, rats were intravenously injected with saline 10 mL/kg using Graseby 3500 micro pump 30 minutes before ischemia; in the Shenfu group, rats were given Shenfu injection (Ya'an Sanjiu Pharmaceutical Co., Ltd., Ya'an, Sichuan Province, China; batch number 010302) 10 mL/kg, 30 minutes before ischemia; in the control group, the superior mesenteric artery was separated but not ligated, with other steps similar to the IR group.

Pathological changes

After the animals were killed, lung tissue was harvested and paraffin sections were created for hematoxylin-eosin staining. The degree of lung injury was evaluated using a modified scoring system [12]. In addition, the left lung was partially removed and immediately cut into 1-mm^3 pieces. The left lung sections were fixed with 2% glutaraldehyde and 1% osmium tetroxide, dehydrated in gradient acetone, embedded in epoxy resin, and sliced into ultrathin slices. Finally, the slices were observed under transmission electron microscopy.

Changes in mean arterial pressure

The mean arterial pressure was monitored 1 hour and 0.5 hours before reperfusion, immediately after reperfusion, and 0.5, 1, 1.5, and 2 hours after reperfusion.

Blood gas test

Left femoral arterial blood (0.5 mL) was harvested after 2 hours of reperfusion and detected for blood gas analysis (i-STAT, Princeton, NJ, USA).

Myeloperoxidase activity and lung wet/dry weight ratio

After animals in each group were sacrificed, the median right lung was frozen at -70°C and myeloperoxidase activity was determined according to the instructions of the detection kit. The lower right lung was weighed with an electronic analytical balance as the wet weight, and then placed in an oven at 60°C for 72
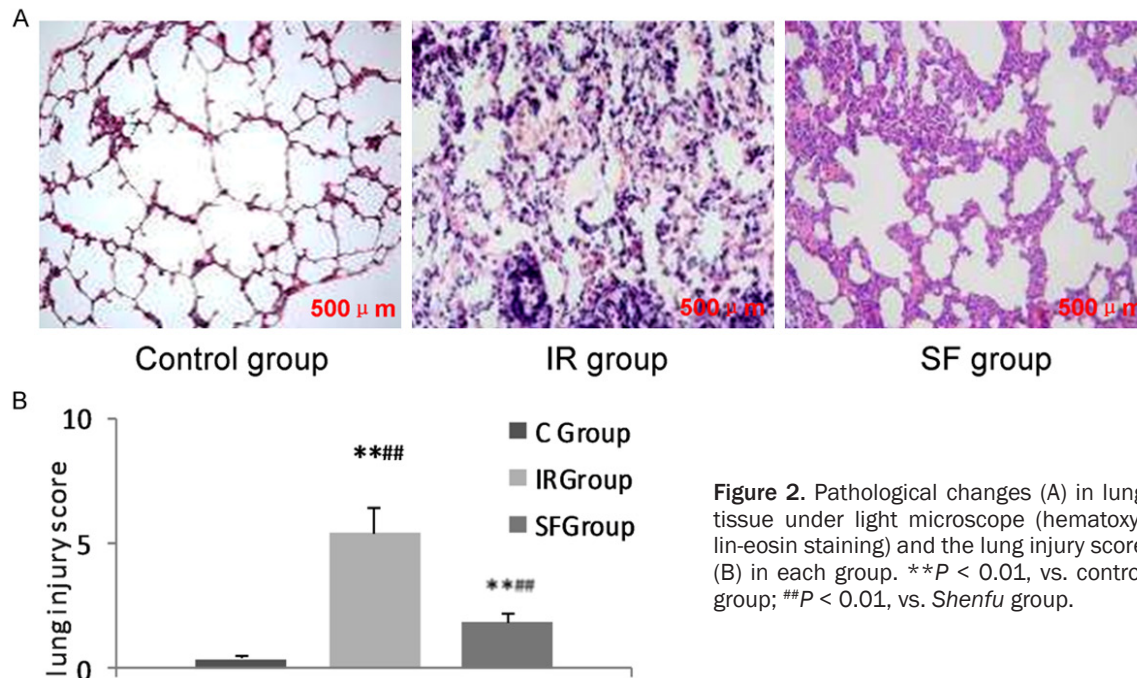
Figure 2. Pathological changes (A) in lung tissue under light microscope (hematoxylin-eosin staining) and the lung injury score (B) in each group. **$P < 0.01$, vs. control group; ##$P < 0.01$, vs. Shenfu group.

hours to measure the dry weight. The lung wet/dry weight ratio was calculated.

TNF-α content in plasma and lung tissue

Blood samples (2 mL) from each group were collected after 2 hours of reperfusion and centrifuged. The supernatant was stored at -70°C. After lung tissue was weighed, it was homogenized with 4°C PBS 10 mL per 1 g wet tissue weight for 30 seconds on ice, and immediately centrifuged at 4°C, 4 500 rpm for 30 minutes. The supernatant was then stored at -70°C for further use. TNF-α content was detected according to the instructions of the ELISA kit (R&D, Minneapolis, MN, USA).

Immunohistochemistry detection of ICAM-1 and iNOS

Lung tissue from each group was subjected to immunohistochemical ABC staining, with brown granules in the cytoplasm being positive product. Twenty-four different visual fields of 12 sections from each group were used (one section in each rat, and two fields in each section) to measure absorbance using an HALPS-2000 medical color image analysis system (Champion Imaging of Tongji Medical University, China). Absorbance was then averaged to obtain a result for each group. Rabbit anti-rat ICAM-1 monoclonal antibody and iNOS polyclonal antibody were provided by Sigma (USA).

Statistical analysis

Data are presented as mean ± standard deviation (x ± s) and analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA). One-way analysis of variance was applied for statistical analysis and independent samples were compared using post hoc (Bonferroni t) testing. $P < 0.05$ was considered statistically significant.

Results

Changes in mean arterial pressure (Figure 1)

The mean arterial pressure was similar in each group before reperfusion ($P > 0.05$) and began to decline in the IR group after reperfusion, and reached a minimum at 2 hours. The mean arterial pressure in the IR group at 30 minutes showed significant differences compared with that in the IR group at other time points ($P < 0.01$). The mean arterial pressure in the *Shenfu* group was significantly higher than that in the IR group at each time point ($P < 0.05$ or $P < 0.01$). At 2 hours after reperfusion, mean arterial pressure was significantly lower in the *Shenfu* group compared with the control group ($P < 0.05$).
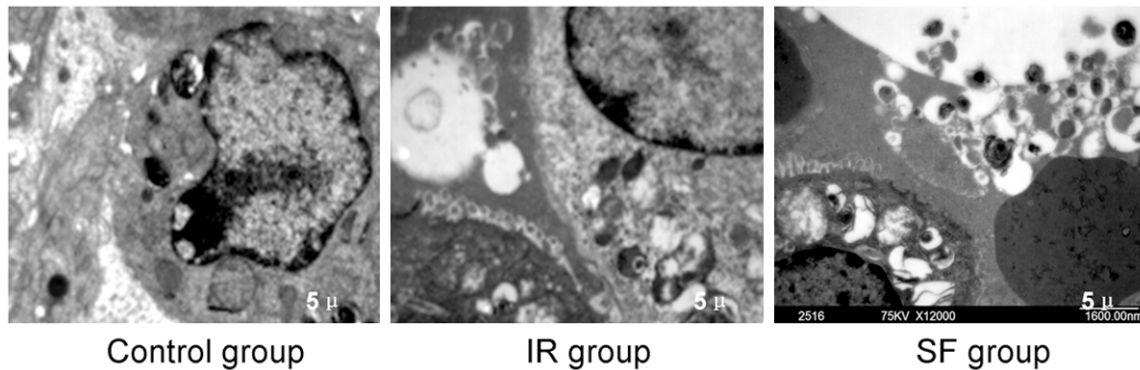
Figure 3. Electron microscopy revealing pathological changes in lung tissue. Intestinal ischemia/reperfusion results in significant lung injury. Degranulation of the pulmonary alveolar type II cells, emptying of osmiophilic lamellar bodies, and cellular ridges lodged and disappeared. Morphological changes in the *Shenfu* group were milder compared with the IR group.

Table 1. Changes in arterial blood gas and lactate in each group (n = 12, x ± s)

Group	pH	PaO_2	$PaCO_2$	Lactate
Control	7.38 ± 0.04	102.4 ± 10.6	39 ± 2.48	1.03 ± 0.26
IR	7.25 ± 0.03**	71.9 ± 11.2**	27 ± 3.38**	2.41 ± 0.42**
Shenfu	7.31 ± 0.03#	87.4 ± 9.6#	32 ± 2.85#	1.75 ± 0.38##

**P < 0.01, *vs.* control group; #P < 0.05, ##P < 0.01, *vs.* IR group.

Table 2. Changes in myeloperoxidase activity and wet/dry weight ratio in each group (n = 12, x ± s)

Group	Myeloperoxidase	Wet/dry weight ratio
Control	0.61 ± 0.09	3.35 ± 0.37
IR	1.26 ± 0.16**	4.79 ± 0.42**
Shenfu	1.08 ± 0.13#	4.16 ± 0.43#

**P < 0.01, *vs.* control group; #P < 0.05, *vs.* IR group.

Table 3. Comparison of tumor necrosis factor-α content in each group (n = 12, x ± s)

Group	Plasma (pg/ml)	Lung tissue (pg/g)
Control	18.6 ± 8.4	499 ± 190
IR	189.7 ± 56.3**	1731 ± 162**
Shenfu	47.5 ± 18.7**,##	683 ± 137##

**P < 0.01, *vs.* control group; ## P < 0.01, *vs.* IR group.

Pathomorphological changes of lung tissue

No abnormalities were found in the control group. In the IR group, interstitial lung edema and polymorphonuclear neutrophil infiltration were apparent, alveolar edema also appeared, and a small amount of bleeding and fibrin exudation was observed. In the *Shenfu* group, no alveolar edema and fibrin exudation was found, and only mild interstitial lung edema and a small amount of polymorphonuclear neutrophil infiltration were observed (**Figure 2A and 2B**).

Ultrastructure of lung tissue under electron microscopy

The ultrastructure of lung tissue was normal in the control group. In the IR group, red blood cells stagnated within the capillaries, inflammatory cells adhered to vascular endothelial cells, type II epithelial cell edema was visible, lamellar bodies appeared degranulated and vacuolized, and their number was also reduced. Nuclear condensation was found, and the perinuclear gap widened. A large number of alveolar neutrophils, mononuclear macrophages and red blood cells left the intravascular space, and the alveolar structure was damaged. In the *Shenfu* group, there were less type II cellular microvilli seen under the electron microscope, and evacuation of osmiophilic plate layer bodies and the extent of damage were reduced (**Figure 3**).

Arterial blood gas analysis

The pH, PaO_2 and $PaCO_2$ in the IR group were significantly decreased (*P* < 0.01), but lactate was significantly increased (*P* < 0.01) compared with the control group. The pH, PaO_2 and $PaCO_2$ in the *Shenfu* group were significantly increased (*P* < 0.05), but lactate was significantly decreased (*P* < 0.01) compared with the IR group (**Table 1**).
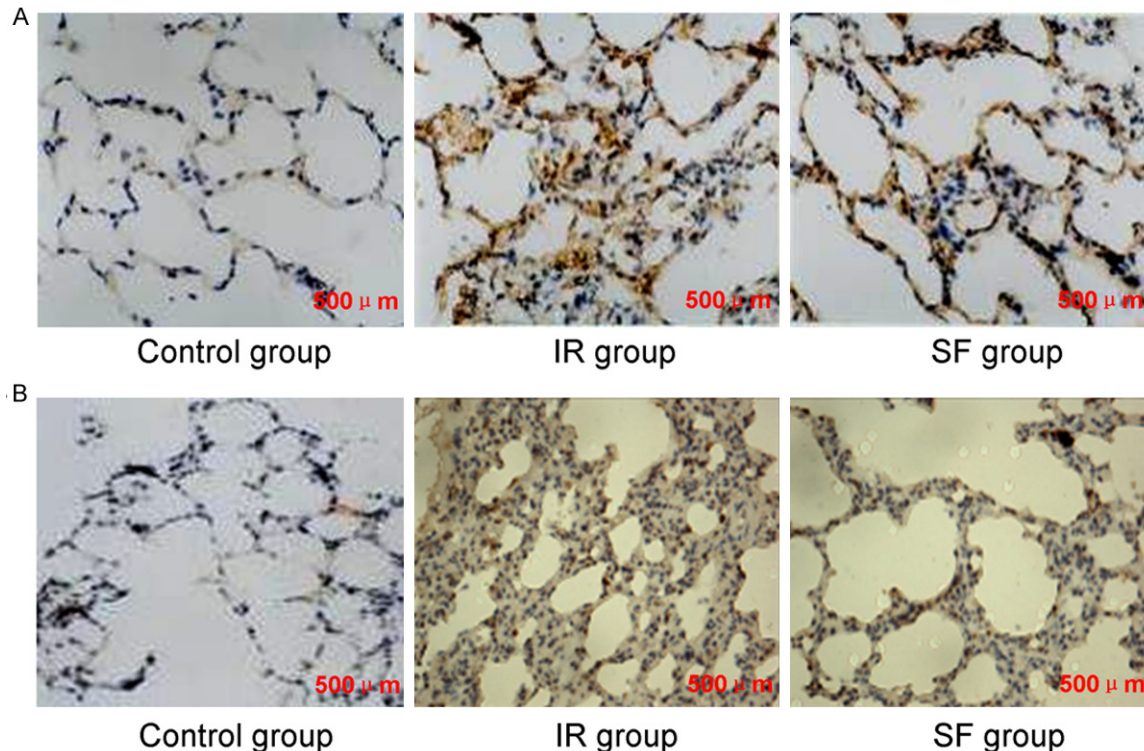
Figure 4. Intercellular adhesion molecule-1 expression A and inducible nitric oxide synthase expression B in lung tissue.

Table 4. Inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1) expression in each group (n = 12, x ± s)

Group	ICAM-1	iNOS
Control	0.1008 ± 0.02021	0.1054 ± 0.01014
IR	0.1369 ± 0.0082**,##	0.1783 ± 0.02563**,##
Shenfu	0.1191 ± 0.0154*	0.1298 ± 0.01865

*$P < 0.05$, **$P < 0.01$, vs. control group; ##$P < 0.01$, vs. Shenfu group.

Changes in lung myeloperoxidase activity and lung wet/dry weight ratio

The level of lung myeloperoxidase reflects the infiltration of neutrophils in lung tissue. Water content in lung is an important indicator of acute lung injury and wet/dry weight ratio is an objective indicator of lung water content. The lung myeloperoxidase activity and lung wet/dry weight ratio in the IR group were significantly higher than those in the control group ($P < 0.01$). After Shenfu injection, myeloperoxidase activity was significantly inhibited and lung wet/dry weight ratio was decreased, with statistical significance compared with the IR group ($P < 0.05$; **Table 2**).

TNF-α content in plasma and lung tissue

The TNF-α content IR group and Shenfu group showed a significant difference compared with the control group (**Table 3**, $P < 0.01$), while TNF-α content in the Shenfu group was lower than that in the IR group (**Table 3**, $P < 0.01$).

ICAM-1 and iNOS expression and distribution in lung tissues

No ICAM-1 or iNOS was positively expressed in the control group (**Figure 4A** and **4B**; **Table 4**). There were a small number of positive cells in the Shenfu group. The number of positive cells was significantly increased in the IR group (**Figure 4A** and **4B**; **Table 4**). The absorbance in the IR group was significantly higher than that in the Shenfu and control groups ($P < 0.05$ or $P < 0.01$).

Discussion

Intestinal IR is a common pathophysiological change in critically ill patients, which not only damages the intestinal tract but also affects the structure and function of remote organs such as lung, even leading to multiple organ

dysfunction syndromes. Intestinal IR is, therefore, considered a leading cause of death in critically ill patients [1, 4]. This study showed that intestinal IR triggered intestinal and lung tissue hemorrhage, edema and neutrophil infiltration, while decreasing mean arterial pressure. This evidence indicated that intestinal IR caused damage to the intestinal mucosal barrier, activated inflammatory mediators, cytokines and polymorphonuclear leukocytes, and triggered a systemic inflammatory response and organ damage, which is characterized by widespread microvascular leakage. In this study, an intestinal IR model was produced through ligation of the superior mesenteric artery. Under the light microscope, alveolar structure was severely damaged in the IR group, with congestion and consolidation visible within alveoli and at alveolar walls. Neutrophils oozed from alveolar space and interstitial tissue, and lung interval was severely thickened. Electron microscopy revealed inflammatory cells adhered to vascular endothelial cells in the IR group, while erythrocytes stagnated within pulmonary capillaries. Type II epithelial cells appeared edematous, lamellar bodies appeared degranulated and vacuolized, and their numbers were reduced. The perinuclear gap widened, and pyknosis was visible. These findings showed that the lung injury model was successful.

The subsequent inflammatory response plays a crucial role in the pathogenesis of lung injury [13-15]. Polymorphonuclear neutrophil accumulation in the lungs is the main feature of intestinal IR-caused lung injury. Polymorphonuclear neutrophils can be activated through adhesion to endothelial cells. The release of oxygen free radicals and proteolytic enzymes are the main contributing factors in multiple organ dysfunction syndrome caused by intestinal IR, while the upregulated expression of local adhesion molecules is the basis of polymorphonuclear neutrophil adhesion and activation [4, 16]. ICAM-1 is responsible for mediating the adhesion and activation of polymorphonuclear neutrophils and endothelial cells, and the main adhesion molecule entering the tissue [17]. Intestinal IR could increase ICAM-1 expression, especially in lung tissue, and ICAM-1 monoclonal antibodies prevent lung injury caused by intestinal IR [17, 18]. The results of this study showed that ICAM-1 expression in lung tissue was significantly increased

in the IR group, and histopathological results found the aggravated injury. In the *Shenfu* group, ICAM-1 expression was significantly reduced compared with the IR group, and lung injury was also significantly attenuated, indicating an inhibitory effect of *Shenfu* on ICAM-1 expression.

After intestinal IR, mucosal barrier function was lost, leading to intestinal bacteria and endotoxin translocation into the systemic circulation [2, 3]. TNF-α *in vivo* is primarily generated by activated macrophages or monocytes, and is one of the strongest stimuli for generation. Accumulating evidence highlighted the contribution of TNF-α in triggering or worsening remote organ damage caused by intestinal IR, and TNF-α is a mediator of intestinal factor-derived lung injury [19, 20]. TNF-α stimulated the release of leukocyte chemotactic factors from vascular endothelial cells, promoted the adhesion and infiltration of polymorphonuclear neutrophils and endothelial cells, and upregulated ICAM-1 expression on endothelial cells. Subsequently, it facilitated polymorphonuclear neutrophil accumulation at the vessel wall, caused damage to endothelial cells, and increased the generation of oxygen free radicals and proteolytic enzymes [20]. The present study showed that the TNF-α content in lung tissue and plasma was significantly increased after intestinal IR, ICAM-1 expression was increased, and intestinal mucosal injury was aggravated. Our findings are consistent with previous studies. *Shenfu* pretreatment significantly inhibits the elevation of TNF-α in plasma and lung tissue, thereby blocking a series of biological effects caused by the synthesis and release of TNF-α. In animal experiments, both anti-TNF-α antibodies and soluble TNF-α receptor were shown to inhibit biological effects mediated by TNF-α [19, 21]. However, clinical applications are potentially dangerous because TNF-α mediates host immune defense response, and blocking its biological activity will inevitably affect the body's immune function. *Shenfu* injection can regulate the immune system, but has no significant immunosuppressive activity. The multi-target effects of *Shenfu* are highly linked with the inhibition of TNF-α activity [7].

Pulmonary microvascular dysfunction and intestinal mucosal barrier damage after intestinal IR are mainly mediated by the increased

expression of iNOS, which promotes NO release. NO then binds with the superoxide anion to produce nitro peroxide. The nitro peroxide is highly cytotoxic and can decrease intracellular ADP levels, thus affecting cellular energy metabolism and enhancing the permeability of epithelial cells. Inhibition of iNOS activity can significantly reduce intestinal mucosa and lung microvascular injury [22, 23]. The present study showed that iNOS expression in lung tissue was increased after intestinal IR, while *Shenfu* injection significantly reduced the increased iNOS expression caused by intestinal IR. This may explain why the mean arterial pressure in the IR group was lower than that in the *Shenfu* group.

Previous studies demonstrated the protective effect of *Shenfu* injection against intestinal IR in rats. This study found that *Shenfu* injection improved oxygenation after IR, reduced inflammatory cytokine (TNF-α, ICAM-1 and iNOS) levels, lowered lung water content, decreased myeloperoxidase activity and pulmonary vascular permeability, and significantly attenuated lung injury. Therefore, we tentatively put forward that *Shenfu* injection inhibits the release of inflammatory cytokines and accordingly protects the lung. Further studies are needed to investigate the mechanism of inhibiting inflammatory cytokines.

In summary, *Shenfu* injection has a significant protection and treatment effect on intestinal IR-induced lung injury. This Chinese herbal preparation has been clinically applied because of a wide range of safe dosage and few side effects [7-11].

Disclosure of conflict of interest

None.

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