Protective effect of Xuebijing injection against acute lung injury induced by left ventricular ischemia/reperfusion in rabbits

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Abstract. Xuebijing (XBJ) is a traditional Chinese medicine preparation. Previous studies have demonstrated that XBJ injection is able to inhibit the uncontrolled release of endogenous inflammatory mediators, attenuate inflammation, and alleviate organ damage. However, there are no relevant reports on the protective effect of XBJ against left ventricular ischemia/reperfusion (I/R)-induced acute lung injury (ALI). Therefore, the aim of the present study was to evaluate the protective effect of XBJ on ALI induced by left ventricular I/R, and provide evidence for the clinical application of XBJ. In the present study, 120 healthy rabbits of mixed gender were randomly assigned to a normal control group, ischemia group, I/R group (I/RG) and XBJ-injection treatment group (TG). In addition, each group was further divided into three subgroups (n=10/subgroup), namely, 30 min pre-ischemia, 30 min post-ischemia and 30 min post-reperfusion subgroups. Blood samples (5 ml) were collected from the jugularis externa and carotis communis of the rabbits at the three time points, and a blood gas analyzer was used to measure the arterial partial pressure of oxygen (PaO₂) and carbon dioxide (PaCO₂). Following sacrifice, the lungs of the rabbits were removed and a bronchoalveolar lavage (BAL) was immediately performed. An enzyme-linked immunosorbent assay was used to measure the expression levels of tumor necrosis factor-α (TNF-α) in the BAL fluid (BALF) and peripheral blood. In addition, the lower lobe of the right lung was removed in order to measure the protein expression levels of intercellular adhesion molecule-1 (ICAM-1) and TNF-α. The results demonstrated that in the rabbits of the TG PaO₂ was increased, PaCO₂ was decreased, the lung tissue congestion edema was attenuated, the expression levels of TNF-α in the peripheral blood and BALF were reduced and the protein expression levels of ICAM-1 and TNF-α in the lung tissue samples were decreased, as compared with those in the I/RG rabbits. These results suggest that XBJ may protect against left ventricular I/R-induced ALI by regulating the expression of the inflammatory mediators TNF-α and ICAM-1.

Introduction

Acute myocardial infarction is a common medical emergency of the cardiovascular system. The clinical treatments for myocardial infarction mainly focus on interventional or thrombolytic therapy. Although interventional or thrombolytic therapy is able to clear the coronary artery and restore blood flow (1), it can also cause reperfusion injury (2). This means that blood reperfusion may occasionally further aggravate the damage (3), and can even cause respiratory dysfunction, with patients with severe cases succumbing to respiratory failure (4). Therefore, the development of drugs that effectively control or reduce ischemia/reperfusion (I/R)-induced lung injury is required. A previous study has found that an imbalance of inflammatory mediators is an important cause of myocardial I/R injury (5). Thus, redressing the balance of inflammatory mediators is an important target for reducing or preventing I/R injury.

Xuebijing (XBJ) is a traditional Chinese medicine preparation (6). In clinical settings, XBJ is combined with antibiotics to treat severe acute lung diseases, including acute lung injury (ALI) (7), sepsis (8) and multiple organ dysfunction syndrome (9). However, to the best of our knowledge, no previous studies have investigated the protective effects of XBJ injection against left ventricular I/R-induced ALI. Therefore, the present study established a rabbit model of left ventricular I/R-induced ALI in order to investigate the underlying pathogenic mechanisms and the protective role of XBJ on respiratory...
function. The following parameters were assessed: The partial pressure of oxygen (PaO_2) and carbon dioxide (PaCO_2); the expression levels of tumor necrosis factor (TNF)-α in peripheral blood and bronchoalveolar lavage (BAL) fluid (BALF); the lung wet/dry weight ratio (W/D); and the protein expression levels of intercellular adhesion molecule-1 (ICAM-1) and TNF-α in the rabbit lung tissue samples.

Materials and methods

**Animals and grouping.** A total of 120 healthy adult (6-7 months old) mixed gender New Zealand rabbits weighing 200-250 g were obtained from the Experimental Animal Center of Zhengzhou University (Zhengzhou, China). Each rabbit was housed in an individual cage (at 20 ± 5°C) under a 12:12 h light and dark cycle for 1 week prior to experimentation. All rabbits had free access to water, and food was removed 8 h prior to the study. The rabbits were randomly divided into four groups (n=30/group), as follows: i) The normal control group (CG); ii) the ischemia group (IG); iii) the I/R group (I/GR); and iv) the I/R+XBJ treatment group (TG). In addition, each group was further divided into three subgroups (n=10/subgroup) as follows: i) 30 min pre-ischemia (T1); ii) 30 min post-ischemia (T2); and iii) 30 min in post-reperfusion (T3). For 12 h prior to the experiment, the rabbits were fasted but had ad libitum access to water. All experimental procedures were approved by the Ethics Committee of Xinxiang Medical University (Xinxiang, China). The present study was conducted in accordance with internationally recognized guidelines on animal welfare (10), as well as local and national regulations.

**Reagents and instruments.** The instruments included a Leica RM2235 microtome (Leica Microsystems GmbH, Wetzlar, Germany), a CR-21G High-Speed Refrigerated Centrifuge (Hitachi, Ltd., Tokyo, Japan), a DGH5033A Enzyme-linked Immunosorbent Assay (ELISA) Analyzer (Shanghai Bogoo Biotechnology, Co., Ltd., Shanghai, China), a fully automated M248 Blood Gas Analyzer (Bayer China Ltd., Shanghai, China) and a BL-420 Biological Signal Collecting and Processing system (Chengdu TME Technology, Co., Ltd., Chengdu, China). The main reagents were as follows: XBJ (10 ml ampoules; Tianjin Chase Sun Pharmaceutical, Co., Ltd., Tianjin, China), 20% urethane (Shanghai Yunjiang Chemical Co., Ltd., Shanghai, China), and ICAM-1 and TNF-α polyclonal antibodies (Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China). Surgical instruments and general laboratory supplies were provided by the Functional Laboratory of Xinxiang Medical University.

**Surgical procedure.** The rabbits underwent general anesthesia with 20% urethane (5 ml/kg) via the ear vein, followed by isolation and intubation of the trachea, right jugularis externa and left carotis communis. Following a thoracotomy, the left anterior descending coronary artery was separated and ligated, with the ST segment elevation arched upward, and with regional myocardial darkening serving as an effective indicator of ischemia. In the CG, the left anterior descending coronary artery was separated but not ligated, and the rabbits were observed for 4 h. In the IG, myocardial ischemia was established by making a slipknot using a 6-0 silk suture around the left anterior descending coronary artery for 1.5 h. In the I/GR, following ligation of the left anterior descending coronary artery for 30 min, the ligature was loosened and the blood supply was restored. The TG underwent the same procedures as the I/GR, and were also given XBJ (10 ml/kg) at two time points: 10 min prior to ischemia and immediately following reperfusion. Prior to the thoracotomy, needle electrodes were inserted into the limbs and chest, and connected to the BL-420 Biological Signal Collecting and Processing system. The electrocardiogram (ECG; paper speed, 50 mm/sec; gain, 1 mV=20 ram) and diaphragm discharge curve were monitored continuously. One end of a lead was connected to a hook positioned on the skin at the point of strongest respiratory movement, and the other end of the lead was connected to a pressure transducer chip connected to the BL-420 system for the recording of respiratory curves.

**Samples.** At the T1, T2 and T3 time points, 5-ml peripheral blood samples were collected from the left common carotid artery, and the PaO_2 and PaCO_2 were measured using the M248 Blood Gas Analyzer. Also, 2-ml venous blood samples were collected from the right external jugular vein for measurement of the level of TNF-α. Subsequently, all rabbits were sacrificed by aeroembolism and the lungs were harvested. BALF samples (5 ml) were collected using Y-type tracheal intubation with one inlet connected to a 50-ml syringe of air, and another connected a 10-ml syringe containing 10 ml 0.9% NaCl. Air and 0.9% NaCl were concurrently introduced into the lungs, and then the 10-ml syringe was pumped back and forth several times to collect the BALF. The lower lobe of the right lung was removed, fixed with paraformaldehyde solution (1 ml/100 g; Qingzhou Hengxing Chemical Co., Ltd., Qingzhou, China), embedded in paraffin (Dekang Medical Instrument Co., Ltd., Xinxiang, China) and cut into 5-μm sections using the Leica RM2235 microtome, prior to being fixed to glass slides coated with (3-aminopropyl)triethoxysilane (Dekang Medical Instrument Co., Ltd.).

**ELISA.** The level of TNF-α in the venous blood and BALF was measured by a double-antigen sandwich ELISA (ELISA: EIA-1126; Beijing Zhongshan Golden Bridge Biological Technology, Co., Ltd.), according to the manufacturer’s protocol. Briefly, each sample (sera; diluted with coating buffer) was applied in triplicate to 96-well plates pre-coated with anti-TNF-α antibody and incubated overnight at 4°C. Following this, the plates were washed three times with phosphate-buffered saline (PBS) and then incubated with biotinylated goat anti-rabbit IgG (1:20,000) for 2 h at 37°C. The plates were then incubated with 0.01% 3,3',5,5'-tetramethylbenzidine (Beijing CellChip Biotechnology Co., Ltd., Beijing, China), after which 2 M H_2SO_4 (Nanjing Taiye Chemical Industry Co., Ltd., Nanjing, China) was added to terminate the reaction. The optical density was recorded at 450 nm using a DG5033A ELISA Analyzer.

**Lung weight and water content.** Lungs were weighed, and then dried in a vacuum drying oven (cat. no. DHG-9420; Shanghai Haixiang Equipment Factory, Shanghai, China) at 80°C for 48 h to obtain the dry weight. The lung wet
weight/dry weight (W/D) ratios of left lung tissue samples were determined, and the water content of the lung tissue was calculated using the following formula: Water content (%) = (wet weight - dry weight)/wet weight x 100.

**Immunohistochemistry.** Lung tissue sections, fixed in formalin (Shanghai Fengshou Biotechnology Co., Ltd., Shanghai, China) and embedded in paraffin, were deparaffinized, routinely rehydrated using graded ethanol, peroxidase-quenched with 3% H$_2$O$_2$, blocked with 5% normal goat serum (Amyjet Scientific, Inc., Wuhan, China) and probed with rabbit anti-rabbit ICAM-1 antibody (final concentration:0.1%; bs-0608R) or rabbit anti-rabbit TNF-α antibody (final concentration:0.1%; bs-2081R) overnight at 4°C. The sections were then thoroughly washed with PBS for 5 min, repeated three times. Subsequently, the tissue sections were incubated with goat anti-rabbit IgG secondary antibody (bs-0295Gs; Beijing Biosynthesis Biotechnology Co., Ltd.) at room temperature for 1 h. The sections were thoroughly washed with PBS for 5 min, repeated three times. Following this, sections were incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-goat antibody (bs-0294R-HRP; Beijing Biosynthesis Biotechnology Co., Ltd.) at 4°C for 30 min. Eventually, reaction products were visualized following diaminobenzidine (DAB) and hematoxylin (Shanghai Yantuo Biotechnology Co., Ltd., Shanghai, China) counterstaining.

The protein expression levels of ICAM-1 and TNF-α in the lung tissue samples of the rabbits at each of the T1, T2 and T3 time points were measured. These experiments were performed in triplicate. According to the results of a semi-quantitative method (11), 10 random fields were selected under a Nikon microscope (80i; Nikon Instruments Co., Ltd, Shanghai China), in which 100 cells were counted. The tissue sections were scored as follows: No staining, ‘¬’; <25% positive cells, ‘+’; 25-75% positive cells, ‘++’; and >75% positive cells, ‘+++’.

**Statistical analysis.** Statistical analyses were conducted using SPSS software, version 11.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. The data were analyzed using an Independent Samples t-test and categorical data were analyzed using a $\chi^2$ test. $P<0.05$ was considered to indicate a statistically significant difference.

**Results**

**Comparison of respiratory curves.** Respiratory curves (Fig. 1) were collected and recorded using the BL-420 Biological Signal Collecting and Processing system. At the T1, T2 and T3 time points, the breathing of the rabbits in the CG was steady and the respiratory amplitude and duration did not significantly
fluctuate. Conversely, the respiratory amplitude for the rabbits in the IG was shallower and the respiratory duration was shorter. In addition, respiratory fluctuations were observed between the three time points. In comparison with the IG, the respiratory amplitude for the rabbits in the I/RG was more shallow and the respiratory duration was shorter. The respiratory curves for the rabbits in the TG resembled those of the rabbits in the CG (Fig. 1).

Comparison of PaO$_2$ and PaCO$_2$. The PaO$_2$ and PaCO$_2$ were measured using the fully automated M248 Blood Gas Analyzer. The PaO$_2$ and PaCO$_2$ of rabbits in the TG were not significantly different from those in the CG at the T1, T2 and T3 time points (P>0.05). The PaO$_2$ values of the rabbits in the IG and I/RG were significantly decreased compared with those in the CG, at the T2 and T3 time points (P<0.001), and particularly decreased at T3 in the I/RG. Furthermore, the PaCO$_2$ values of the rabbits in the IG and I/RG were significantly increased compared with those in the CG, at the T2 and T3 time points (P<0.001; Fig. 2).

Comparison of W/D value and water content in the left lung tissue samples of the rabbits. At the T1, T2 and T3 time points, the W/D weight ratio and water content of the left lung tissue from the CG did not significantly differ (P>0.05). Conversely, those of the tissue from the IG and I/RG were significantly increased (P<0.001) compared with those in the CG, with the greatest increases being observed for the I/RG. The W/D weight ratio and water content of the left lung tissue from the TG were not significantly different from those in the CG at any of the three time points (P>0.05; Fig. 3).

General observation of the rabbit lungs at T3. At the T3 time point, the lungs of rabbits in the CG were light pink and small in size. In the IG, the lungs appeared dark red and small, with an uneven surface and visible hemorrhages. Similarly, congestion and pulmonary edema were apparent in the lungs of the I/RG, and the volume was larger, the margins of the lungs were blunt, the color was uneven and clear spot bleeding and bruising was observed in the lower lobe of the lungs. In addition, white foamy/bloody liquid was overflowing from the lung.
section. Conversely, the TG lungs exhibited only mild edema, appeared red in color and had a smooth surface.

**Light microscopy observations of the rabbit lungs at T3.** At the T3 time point, the alveolar structure of the lungs in the CG group was clear, the walls of the alveolae were thin and no inflammatory cells had infiltrated into the alveolar space and lung interstitium. In the IG, pulmonary vascular congestion, focal alveolar hemorrhages and small focal alveolar collapse/atelectasis were observed in some areas of the lung. In the I/RG, dilatation and congestion was observed in the pulmonary capillaries, the alveolar septum was thickened, a large number of leukocytes had infiltrated and aggregated and hemorrhage and exudation were detected in the alveolar cavity. In addition, atelectasis was observed in some alveolae. Conversely, in the TG, the alveolar structure was relatively complete and hemorrhage and exudation of the alveolar was markedly reduced, as compared with the I/RG lungs, although alveolar atrophy and infiltrated inflammatory cells were infrequently observed (Fig. 4).

**Expression levels of TNF-α in the BALF and the peripheral blood.** The expression levels of TNF-α in the BALF and peripheral blood from the four groups were detected by performing an ELISA. At the T1, T2 and T3 time points, there was no significant difference in the expression levels of TNF-α in the peripheral blood and BALF between the CG and TG (P>0.05). In the IG, the expression levels of TNF-α in the peripheral blood and BALF were increased at T2, and reached a maximum at T3 (P<0.001). At T2 and T3, the expression levels of TNF-α in the peripheral blood and BALF in the TG were significantly lower compared with those in the IG and I/RG (P<0.001; Fig. 5).

**Protein expression of ICAM-1 and TNF-α in the rabbit lung tissue samples at T3.** The immunoreactive product of ICAM-1 was brown and was observed in the membrane and cytoplasm. TNF-α formed dark brown-to-yellow granules that were observed in the cytoplasm. As shown Figs. 6 and 7, there was an evident increasing trend in the expression of ICAM-1 and TNF-α from CG and IG to I/RG at T3, and the levels of ICAM-1 and TNF-α in the TG were comparable to those in the CG and markedly lower than those in the IG and I/RG.

**Comparison of the association between the protein expression levels of ICAM-1 and TNF-α in the rabbit lung tissue samples at T3.** The association between the protein expression levels of ICAM-1 and TNF-α in the rabbit lung tissue samples at T3 was investigated. A positive correlation between the protein expression levels of ICAM-1 and TNF-α was observed (Table I).

**Discussion**

Due to the structural characteristics of the left anterior descending coronary artery, the blood supply of the heart is prone to ischemia (12-14), and the recovery of blood perfusion to the ischemic myocardium is an important therapeutic strategy that reduces the occurrence of ischemic injury. However, in some cases reperfusion may further aggravate damage or induce irreversible damage; this phenomenon is called reperfusion injury. As an important organ with roles in immunity and metabolism, the lung has a high probability of being affected by reperfusion injury and is prone to inflammation (15).

Previous studies have demonstrated that the activation of a large number of neutrophils occurs in the early stages of ischemia (16), and that these neutrophils enhance the process of reperfusion (17). In addition, activated neutrophils promote the
JI et al: PROTECTIVE EFFECT OF XUEBIJING INJECTION ON I/R-INDUCED ACUTE LUNG INJURY

Figure 5. Expression levels of TNF-α in the peripheral blood and BALF in the four groups at the T1, T2 and T3 time points. The expression levels of TNF-α in the (A) peripheral blood and (B) BALF. Data are presented as the mean ± standard deviation. ΔP<0.001, at the same time vs. the CG; #P<0.001, the same group vs. T1; *P<0.001, the same group vs. T2; **P<0.001, at the same time vs. the IG and I/RG. CG, normal-control group; IG, ischemia group; I/RG, ischemia/reperfusion group; TG, treatment group; T1, 30 min pre-ischemia; T2, 30 min post-ischemia; T3, 30 min post-reperfusion; BALF, brochoalveolar lavage fluid.

Figure 6. Expression of intercellular adhesion molecule-1 in lung tissue samples of rabbits in the (A) normal-control, (B) ischemia, (C) ischemia/reperfusion and (D) treatment groups 30 min post-reperfusion following immunohistochemical (diaminobenzidine) staining. Magnification, x400.

Figure 7. Protein expression of tumor necrosis factor-α in lung tissue samples of rabbits in the (A) normal-control, (B) ischemia, (C) ischemia/reperfusion and (D) treatment groups 30 min post-reperfusion following immunohistochemical (diaminobenzidine) staining. Magnification, x400.
the expression levels of ICAM-1 has a key role in neutrophil aggregation, activation and the excessive release of proinflammatory mediators, including TNF-α. The excessive release of TNF-α in turn induced the overexpression of ICAM-1, which led to the adhesion of neutrophils to endothelial cells, eventually leading to lung inflammatory injury. XBJ exhibited a protective effect in the rabbit lung tissue samples, inhibited the excessive release of early inflammatory cytokines, including TNF-α, and decreased the expression levels of ICAM-1.

In conclusion, the present study investigated the effect of XBJ on left ventricular I/R-induced ALI by performing a blood gas analysis and measuring the expression levels of ICAM-1 and TNF-α. The results demonstrated that, following treatment with XBJ, the release of the inflammatory mediators ICAM-1 and TNF-α was effectively inhibited, and the PaO₂ was improved. Understanding the molecular mechanism underlying the anti-inflammatory effect of XBJ will provide a theoretical basis and experimental evidence for its clinical application.

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Table I. Comparison of the association between the protein expression levels of ICAM-1 and TNF-α in the lung tissue samples of rabbits in the 4 groups at the T3 time point.

|       | CG   | IG   | I/RG | TG   |
|-------|------|------|------|------|
| -     | 6    | 2    | 0    | 4    |
| +     | 1    | 0    | 3    | 1    |
| ++    | 0    | 2    | 1    | 0    |
| +++   | 0    | 0    | 2    | 0    |

ICAM-1, intercellular adhesion molecule-1; TNF-α, tumor necrosis factor-α; CG, normal-control group; IG, ischemia group; I/RG, ischemia/reperfusion group; TG, treatment group; T3, 30 min post-reperfusion; ‘−’, no positive cells; ‘+’, <25% positive cells; ‘++’, 25-75% positive cells; ‘+++’, >75% positive cells.

release of large amounts of inflammatory cytokines, including TNF-α (18,19), interleukin (IL)-6 (20,21), IL-10 (22-24) and ICAM-1 (25), in order to expand the inflammatory response. ICAM-1 has a key role in neutrophil activation, aggregation and the release of inflammatory mediators (26-28). Under normal circumstances, the protein expression levels of ICAM-1 are negligible, and this prevents pathological damage to the body (29). However, during I/R and other pathological conditions, the expression of proinflammatory mediators is stimulated and the expression levels of ICAM-1 are increased significantly (30). This in turn promotes the adherence of neutrophils to endothelial cells and their migration across the endothelial barrier, resulting in inflammation and various pathological changes that characterize reperfusion injury (7).
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