

## Levosimendan Reduces Lung Injury in a Canine Model of Cardiopulmonary Bypass

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**Background and Objectives:** To explore the lung-protective effect of levosimendan (LS) during cardiopulmonary bypass in a canine model by determining the wet/dry weight (W/D) ratio of lung tissue, malonaldehyde (MDA) and superoxide dismutase (SOD) concentrations, and performing a histological evaluation.

**Materials and Methods:** Thirty-two canines were divided randomly into four groups and underwent a routine aortic cross-clamping cardiopulmonary bypass procedure for 1 h, followed by recovery for 2 h. Animals were handled as follows: group C (means control group), no special treatment after aortic cross clamping; group P (means pulmonary artery perfusion group), pulmonary artery perfusion with cold oxygenated blood after aortic cross clamping; group LSIV (means intravenous injection of LS group), intravenous injection of LS (65 µg/kg) before thoracotomy, and the rest of the procedure was identical to the control group; group LPS (means pulmonary perfusion with LS group), pulmonary perfusion with cold oxygenated blood combined with LS (65 µg/kg) after aortic cross clamping. Lung tissues were removed and subjected to evaluation of pathological alterations, W/D ratio and MDA and SOD concentrations.

**Results:** In group C, the W/D ratio and MDA concentration were higher, while the SOD concentrations were lower ( $p < 0.05$ ). Compared with groups P and LSIV, the MDA concentration was lower in group LPS, while that of SOD was higher ( $p < 0.05$ ); Light and electron microscopy indicated that LS intervention reduced impairment of lung tissues.

**Conclusion:** Our findings suggest that LS plays an important role in protecting lung tissues. (Korean Circ J 2016;46(3):402-407)

**KEY WORDS:** Levosimendan; Cardiopulmonary bypass; Canine; protective effect.

### Introduction

Cardiopulmonary bypass (CPB) technology was a milestone in the development of cardiovascular surgery, and made cardiac surgery safer and feasible, enabling effective treatment of various

cardiovascular diseases. However, impairment of organs is a serious problem during CPB; the lung tends to experience the most severe injury. Almost all patients receiving CPB during cardiac surgery suffer lung injuries, ranging from mild subclinical symptoms to severe acute respiratory distress syndrome, which leads to longer hospital stay and a higher mortality rate.<sup>1,2)</sup> The mechanism of lung injury by CPB is complex and unclear. Lung injury is correlated with CPB-induced pulmonary ischemia reperfusion injury and the systemic inflammatory response; indeed, ischemia reperfusion injury plays the predominant role.<sup>3,4)</sup> Levosimendan (LS), a novel calcium sensitizer, enhances myocardial contractility without increasing the intracellular concentration of calcium or oxygen consumption. LS is thought to play an important role in protecting organs. This study aimed to explore the lung-protective effect of LS during CPB in a canine model.

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## Materials and Methods

### Materials

#### Animals

A total of 32 healthy canines of body weight  $12 \pm 1.94$  kg were selected for this study without gender restriction (Animal Experiment Center, Anhui Medical University).

#### Reagents and equipment

Levosimendan (Qilu Pharmaceutical Co., Ltd., Ji Nan, China); malonaldehyde and superoxide dismutase reagent kits (Nanjing Jian Cheng Bioengineering Institute, Nan Jing, China); small animal ventilator (Chengdu Taimeng Science And Technology Co., Ltd., Cheng Du, China);  $-80^{\circ}\text{C}$  cryogenic refrigerator (Sanyo, Osaka, Japan); light microscope (CX40, Olympus, Tokyo, Japan); electron microscope (Hitachi, Tokyo, Japan); electronic balance (Sartorius, Gottingen, Germany); JHF1-DHG-9076A electrothermal constant temperature dry box (Beijing Tai'an Technology Services Ltd., Beijing, China); WI313182 blood-gas analyzer (East & West Analytical Instrument Co., Ltd., Beijing, China); cardiopulmonary bypass (Terumo Cardiovascular Systems Corporation, Ann Arbor, MI, USA); and CPB supporting tubes; CPB catheters and tracheal tubes.

### Methods

#### Model and divisions

A total of 32 canines were divided randomly into four groups of eight animals per group. Animals in group C (control group) did not undergo any special procedure during CPB animals in group P underwent pulmonary artery perfusion with cold oxygenated blood after blocking of the aorta (oxygenated blood taken from the oxygenator was bathed in ice water and then maintained at  $0-4^{\circ}\text{C}$ ). The pulmonary artery perfusion parameters were as follows: perfusion volume, 40-50 mL/kg.min; perfusion pressure, 30 mmHg; and perfusion duration, 8-10 min. Animals in group LSIV underwent

LS injection ( $65 \mu\text{g}/\text{kg}$ ) before blocking of the aorta; the remaining procedure was identical to that for group C. Animals in group LSP underwent pulmonary artery perfusion with low-temperature oxygenated blood containing LS ( $65 \mu\text{g}/\text{kg}$ ) (oxygenated blood taken from the oxygenator was bathed in ice water and maintained at  $0-4^{\circ}\text{C}$ ); the perfusion conditions were identical to those in group P. Canines in each group were anesthetized intravenously with pentobarbital ( $30 \text{ mg}/\text{kg}$ ) and ventilated mechanically (tidal volume 12 mL/kg, oxygen concentration 45%, and breathing frequency 25/min). Vital signs were monitored. Punctures of the femoral artery and vein were performed for arterial pressure measurement and transfusion, respectively. Mid-sternal thoracotomy was elected and CPB was established as a routine procedure by intubation of the ascending aorta and right atrium. Following completion of CPB the aorta was blocked, and myocardial preservation solution was perfused every 30 min. After 1 h, the aorta was reopened for heart resuscitation. Mechanical ventilation was restarted, and CPB was continued for 2 h. Finally, right lung tissues were removed and canines were euthanized by hyperkalemia injection.

#### Sample handling

Lung tissues were immersed in formaldehyde for 24 h. The tissues were then embedded in wax, sectioned and stained with hematoxylin and eosin for light microscopy observation. Removed fresh lung tissues of right lung inferior lobe ( $0.8 \times 0.8 \times 0.8$  mm) were immersed in glutaraldehyde for electron microscopy observation. A portion of the tissue of the middle lobe of the right lung was preserved for measurement of the wet/dry weight (W/D) ratio; the remainder was stored in a cryogenic refrigerator at  $-80^{\circ}\text{C}$  for determination of malonaldehyde (MDA) and superoxide dismutase (SOD) concentrations.

#### Wet/dry weight ratio of lung tissue

The wet weights of portions of the right lung tissues were determined, placed into an  $80^{\circ}\text{C}$  oven for 24 h, and their dry weight measured. The W/D ratios of lung tissues were then calculated.

**Table 1.** Wet/dry weight ratio, malonaldehyde content and superoxide dismutase activity

| Group | n | Wet/dry weight ratio | Malonaldehyde (nmol/mgprot) | Superoxide dismutase (U/mgprot) |
|-------|---|----------------------|-----------------------------|---------------------------------|
| C     | 8 | $10.05 \pm 0.56^*$   | $64.08 \pm 5.78^*$          | $105.30 \pm 8.68^*$             |
| P     | 8 | $7.50 \pm 0.32$      | $24.37 \pm 4.63$            | $198.21 \pm 10.11$              |
| LSIV  | 8 | $7.93 \pm 0.42$      | $24.31 \pm 4.20$            | $203.26 \pm 14.44$              |
| LSP   | 8 | $7.28 \pm 0.58$      | $6.61 \pm 0.91$             | $305.04 \pm 10.25$              |

Data are expressed as means  $\pm$  standard deviation. One-way analysis of variance was performed to evaluate the differences in mean W/D ratios and SOD concentrations among the groups. The Kruskal-Wallis test was performed to assess the differences in MDA concentrations among the groups. \* $p < 0.05$ . W/D: wet/dry weight, MDA: malonaldehyde, SOD: superoxide dismutase, C: control group, P: pulmonary artery perfusion group, LSIV: intravenous injection of LS group, LSP: pulmonary perfusion with LS group

*Measurements of malonaldehyde and superoxide dismutase*

Stored lung tissues were thawed and made into tissue homogenates for determination of MDA and SOD concentrations in accordance with the manufacturer's instructions.

**Statistical analysis**

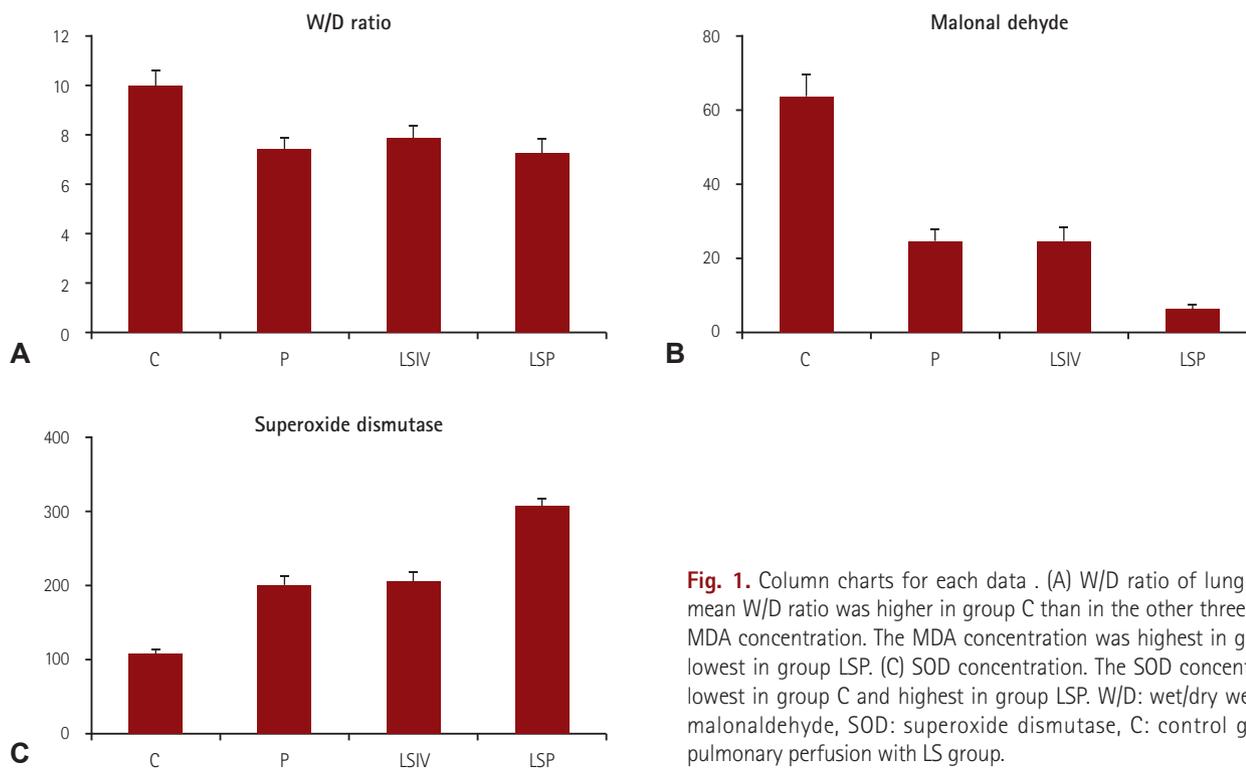
Data were expressed as means±standard deviation. Significance for statistical tests was predetermined at  $p < 0.05$ . The normality of

distributions was evaluated by one-sample Kolmogorov-Smirnov test after testing the homogeneity of variances. One-way analysis of variance was performed to evaluate differences in mean W/D ratios and SOD concentrations among the groups, followed by the Student-Newman-Kuels method. Then, the Kruskal-Wallis test was performed to evaluate the differences in MDA concentrations among the groups. For pair-wise comparisons, a Mann-Whitney U test was applied and  $p$  values were corrected using Bonferroni

**Table 2.** Wet/dry weight ratios, and malonaldehyde and superoxide dismutase concentrations

| Group        | Wet/dry weight ratio |         | Malonaldehyde (nmol/mgprot) |       | Superoxide dismutase (U/mgprot) |  |
|--------------|----------------------|---------|-----------------------------|-------|---------------------------------|--|
|              | p                    | Z value | p                           | p     |                                 |  |
| C vs. P      | <0.05                | -3.361  | <0.001                      | <0.05 |                                 |  |
| C vs. LSIV   | <0.05                | -3.361  | <0.001                      | <0.05 |                                 |  |
| C vs. LSP    | <0.05                | -3.361  | <0.001                      | <0.05 |                                 |  |
| P vs. LSIV   | NS                   | -0.105  | 0.959                       | NS    |                                 |  |
| P vs. LSP    | NS                   | -3.361  | NS                          | <0.05 |                                 |  |
| LSIV vs. LSP | NS                   | -3.361  | NS                          | <0.05 |                                 |  |

One-way analysis of variance was performed to evaluate the differences in mean W/D ratios and SOD concentrations among the groups, followed by the Student-Newman-Kuels method. The Kruskal-Wallis test was performed to evaluate the differences in MDA concentrations among the groups. For pair-wise comparisons, the Mann-Whitney U-test was applied. A  $p < 0.05$  was considered to indicate statistical significance. W/D: wet/dry weight, MDA: malonaldehyde, SOD: superoxide dismutase, C: control group, P: pulmonary artery perfusion group, LSIV: intravenous injection of LS group, LSP: pulmonary perfusion with LS group



**Fig. 1.** Column charts for each data. (A) W/D ratio of lung tissue. The mean W/D ratio was higher in group C than in the other three groups. (B) MDA concentration. The MDA concentration was highest in group C and lowest in group LSP. (C) SOD concentration. The SOD concentration was lowest in group C and highest in group LSP. W/D: wet/dry weight, MDA: malonaldehyde, SOD: superoxide dismutase, C: control group, LSP: pulmonary perfusion with LS group.

adjustment. Six multiple comparisons were performed and a value of  $p < 0.0083$  was considered to indicate significance.

## Results

### Wet/dry weight ratio of lung tissue

Compared with group C, the W/D ratios of groups P, LSIV and LSP were significantly lower ( $p < 0.05$ ). There were no significant differences in W/D ratios among groups P, LSIV and LSP (Fig. 1A, Tables 1 and 2).

### Measurement of malonaldehyde concentration

Compared with group C, the MDA concentrations in groups P, LSIV and LSP were significantly lower ( $p < 0.05$ ). Compared with groups P and LSIV, the MDA concentration was significantly lower in group LSP ( $p < 0.05$ ). There was no significant difference in MDA concentration between groups P and LSIV (Fig. 1B, Tables 1 and 2).

### Measurement of superoxide dismutase concentration

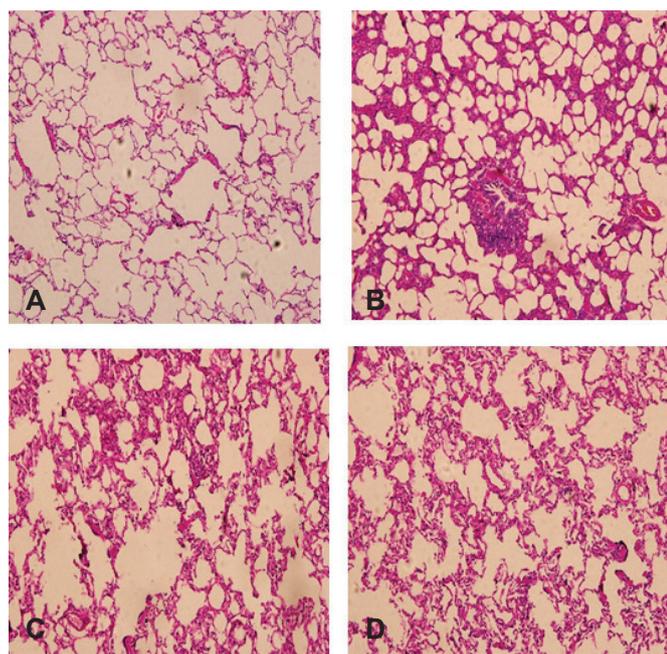
Compared with group C, the SOD concentrations in groups P, LSIV

and LSP were significantly higher ( $p < 0.05$ ). Compared with groups P and LSIV, the MDA and SOD concentrations in group LSP were significantly higher ( $p < 0.05$ ). There was no significant difference between groups P and LSIV (Fig. 1C, Tables 1 and 2).

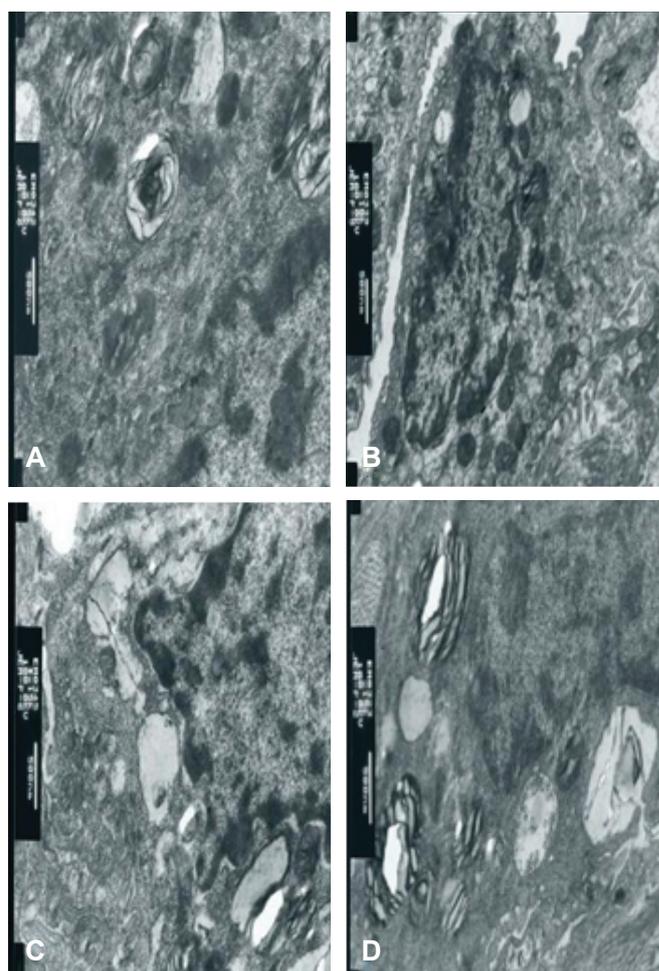
### Pathological alterations

#### Light microscopy observations

Edema of intercellular fluid, infiltration of inflammatory cells and destroyed alveolar space containing massive numbers of erythrocytes and inflammatory cells and large quantities of serous



**Fig. 2.** Light micrograph of lung tissues (HE staining of lung tissues,  $\times 100$ ). (A) group LSP, (B) group C, (C) group P, and (D) group LSIV. As shown in Fig. 1, edema of intercellular fluid, infiltration of inflammatory cells and destroyed alveolar space containing massive numbers of erythrocytes and inflammatory cells and large amounts of serous exudate were found in groups C and LSIV. Compared with group C, edema, inflammatory infiltration, hemorrhage and exudate in groups P and LSP were significantly ameliorated. LSP: pulmonary perfusion with LS group, C: control group, P: pulmonary artery perfusion group, LSIV: intravenous injection of LS group.



**Fig. 3.** Electron micrographs of lung tissues (electron microscopy observation of type II epithelial cells,  $\times 20000$ ). (A) group LSP, (B) group C, (C) group P, and (D) group LSIV. As shown in Fig. 2, mitochondria of II type epithelial cells were swollen and the electron density of the mitochondrial matrix was decreased in groups C, LSIV and LSP. Structures exhibited chaotic ridges, vacuolization of lamellar bodies, fewer microvilli in the cell membrane and nuclear pleomorphism. However, these alterations were significantly ameliorated in groups LSP and P. LSP: pulmonary perfusion with LS group, C: control group, P: pulmonary artery perfusion group, LSIV: intravenous injection of LS group.

exudate were present in groups C and LSIV. In both of these groups, the structure of alveoli was destroyed, as exemplified by collapse and compensatory emphysema. Compared with group C, edema, inflammatory infiltration, hemorrhage and exudate in groups P and LSP were significantly ameliorated. Moreover, less tissue injury was found (Fig. 2).

#### *Electron microscopy observation*

Mitochondria of II type epithelial cells were swollen and the electron density of the mitochondrial matrix was decreased in groups C, LSIV and LSP. Structures exhibited chaotic ridges, vacuolization lamellar body, fewer microvilli in the cell membrane and nuclear pleomorphism. However, these alterations were significantly ameliorated in groups LSP and P (Fig. 3).

## Discussion

CPB is thought to be essential for cardiac surgery. However, within the lungs is reduced or even non-existent during aortic clamping when CPB is activated, and little blood is supplied by the bronchial artery. This leads to ischemia and hypoxia, reducing production of both adenosine triphosphate (ATP) and alveolar surfactant, and increasing lung vascular permeability, protein exudation, and pneumonema.

When pulmonary circulation recovers, a large quantity of oxygen free radicals is produced by xanthine oxidase, which is activated by the p38-mitogen activated protein kinase (p38MARK) pathway under hypoxia, leading to intensive lipid peroxidation. Therefore, MDA, as a marker of lipid peroxidation, and other aldehydes are generated, which altered the functions of biomacromolecules and destroy the structure of nucleic acids and chromosomes. The oxygen free radicals activated during ischemia reperfusion exhaust antioxidants such as SOD. MDA and SOD are effective markers of injury during ischemia reperfusion of lung tissues.

A protective effect on the lungs of perfusion with cold oxygenated blood into the pulmonary artery during CPB has been confirmed clinically. In accordance with our findings, compared with the control group, the W/D ratio and MDA concentration were lower, while the SOD concentration was significantly higher, in the perfusion groups. Pathologically, edema and inflammatory exudate in lung tissue were reduced, and damage to the mitochondria of type II pneumocytes was ameliorated. The possible explanations include: 1) removal of activated neutrophils from pulmonary vessels by the mechanical effect of perfusion, which decreases aggregation of inflammatory cells and release of inflammatory mediators; 2) perfusion with cold oxygenated blood reduces

lung temperature, leading to decreased consumption of ATP and generation of oxygen radicals within lung tissues, indicative of positive adjustment of lung metabolism under hypoxia; and 3) prevention of cellular edema by increasing the colloid osmotic pressure of pulmonary vessels.<sup>5-8)</sup>

LS, a new calcium sensitizer that increases the sensitivity of troponin to  $Ca^{2+}$ , has been applied to enhance myocardial contractility without increasing intracellular calcium concentration and oxygen consumption. LS plays a role in organ protection by resisting ischemia reperfusion injury, cellular apoptosis and decreasing levels of inflammatory factors. In our study, compared with the control group, the W/D ratio and MDA concentration were lower, while the SOD concentration was significantly higher, in the LSIV group. Pathologically, edema and inflammatory exudate in lung tissues were reduced and damage to mitochondria of type II pneumocytes was ameliorated. Gecit et al.<sup>9)</sup> reported similar results in a mouse model of renal ischemia reperfusion, which suggested that LS prevents ischemia reperfusion injury by inhibiting lipid peroxidation and decreasing levels of reactive oxygen species. Additionally, compared with the LSIV and P groups, lung protection was superior in the LSP group. There are two possible reasons for this finding: 1) LS is distributed more equally within alveoli following pulmonary artery perfusion, which results in greater treatment efficacy<sup>8)</sup>; and 2) protective effects of both LS and pulmonary artery perfusion. The non-significant difference between the LSIV and P groups suggested that the protective effect of intravenous injection of LS is similar to that of pulmonary artery perfusion. However, whether intravenous injection or pulmonary artery perfusion is superior is unclear; therefore, further research is warranted.

Much research has focused on the mechanism underlying the protective effect of LS on organs. Enhanced phosphorylation of p38-MARK by LS can prevent ischemia reperfusion by decreasing levels of oxygen free radicals.<sup>10)</sup> Also, in patients suffering heart failure, LS exerts an anti-inflammatory effect by decreasing levels of Interleukin-6 (IL-6) and tumor necrosis factor.<sup>11)</sup> The predominant hypothesis is an increase in the number of activated mitochondrial ATP-sensitive  $K^+$  channels (mito $K_{ATP}$  channel) by LS. Both glibenclamide and 5-hydroxydecanoate, which are mito $K_{ATP}$  channel inhibitors, can inhibit the myocardial protective effect of LS.<sup>12)13)</sup> Therefore, there is a correlation between the lung-protective effect of LS and activated mito $K_{ATP}$  channels. Perfusions containing LS, based on its myocardial protective effect, can inhibit apoptosis of myocardial cells and upregulate the expression of bcl-2 gene, also resulting in inhibition of apoptosis.<sup>14)</sup> This suggests that the lung-protective effect of LS is mediated by inhibition of apoptosis.

In our study, pneumonema progression and MDA and SOD concentrations in lung tissues were ameliorated significantly in the

groups in which LS was administered, which indicated that LS can protect lung tissues. The mechanism of this protection is unclear, although the p38MAPK pathway, activated mitoK<sub>ATP</sub> channels and inhibition of apoptosis may be involved. A more effective method of protecting the lung during CPB was developed in this study; however, further research is required.

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