

The effect of aprotinin on ischemia–reperfusion injury in an in situ normothermic ischemic lung model

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Abstract

Objectives: In the context of the physiopathology of damage due to ischemic preservation and reperfusion injury following preservation, we aimed to demonstrate the positive effects of the addition of aprotinin, a serine protease inhibitor, to low potassium dextran (LPD), used as a single-flush solution in normothermic ischemic animal models, on lung protection and the prevention of reperfusion injury. **Methods:** In the study, 21 New Zealand white rabbits were used as experimental subjects. The subjects were ventilated with the assistance of a manual mechanical ventilator at 30 breaths/min and 10 ml/kg tidal volume. Lung protection solution was supplied to the pulmonary artery via a catheter. After applying the solution, ischemia was carried out for 120 min. At the end of this period, reperfusion was carried out for 90 min. The subjects were divided into three groups of seven subjects each. In the control group, pulmonary perfusion solution was not employed, whereas in the second group LPD was employed, and in the third group LPD and aprotinin (LPD + A) were perfused. Blood gas analysis, bronchoalveolar lavage (BAL) fluid examination, tissue malondialdehyde (MDA) level analysis and morphological examinations were performed. **Results:** The LPD + A group showed the significantly highest levels of oxygenation at the 15th and 60th minutes of reperfusion (297 ± 76.7 and 327 ± 97.4 mmHg) in comparison to the LPD (157 ± 20.6 and 170 ± 53.6 mmHg) and control (64 ± 8.4 and 59 ± 7.2 mmHg) groups ($P < 0.001$). The LPD + A group showed the significantly lowest levels of alveolar-arterial oxygen difference at the 60th minute of reperfusion (389 ± 15 mmHg) in comparison to the LPD (478 ± 19 mmHg) and control (542 ± 23) groups ($P < 0.001$). The BAL fluid neutrophil percentage was significantly lower in the LPD + A group ($22 \pm 2.4\%$) compared to the LPD ($31 \pm 6.1\%$) and control ($38 \pm 2.4\%$) groups. MDA levels were significantly lower in the LPD + A group (119.8 ± 5.3 nmol MDA/g) when compared to the LPD (145.06 ± 9.5 nmol MDA/g) and control (147.3 ± 3.9 nmol MDA/g) groups ($P < 0.05$). Morphological examinations revealed pathological lesions and alveolar hemorrhaging in all samples, with the LPD + A group having statistically more significant levels than the LPD and control groups ($P < 0.005$). The LPD + A group had a significantly lower percentage of pathological lesions and alveolar hemorrhage grade values than the LPD and control groups ($P < 0.005$). **Conclusions:** It was observed that the addition of aprotinin to LPD solution as a pulmonary flush solution in an in situ normothermic ischemic lung model prevents reperfusion injury by means of various mechanisms and also protects the morphological, functional and biochemical integrity of the lung. In our view, therefore, the addition of aprotinin to lung protection solution will provide positive results in lung transplantation protocols. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Ischemia–reperfusion injury; Lung; Aprotinin

1. Introduction

Lung protection research not only aims to prolong the safe ischemia period but also to increase the quality of protection [1]. Cell and tissue damage occurs in organs

temporarily exposed to ischemia and which remain under hypoxia. With the correction of ischemia, additional damage develops due to reperfusion. This complex cycle, consisting of at least two components, is called ischemia–reperfusion injury [2,3]. The causes of early graft dysfunctions are mostly insufficient lung protection and direct cell injury originating from ischemia–reperfusion injury. Damage at reperfusion is much greater, and studies have shown that less injury develops in 3 h ischemia than in 1 h reperfusion [4,5].

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In the last few years, the main research goal in lung transplants has been to identify the main cellular and molecular basis of ischemia–reperfusion injury. Recent research has shown that ischemia–reperfusion injury depends on a chain of complex events such as free oxygen radical formation, neutrophil sequestration, lysosomal and proteolytic enzyme activation, complement activation and endothelial cell damage [6–8]. For this reason, aprotinin, a serine protease inhibitor that is in wide clinical use to minimize perioperative blood loss in cardiac operations, may be ideally suited to reduce the effects of lung reperfusion injury since it also suppresses the release of lysosomal enzymes and inhibits their actions [8]. However, there is no consensus on ideal perfusion content, method of application, addition of suitable pharmacological agents or provision of suitable conditions.

In this study, we examined the effects of aprotinin, a serine protease inhibitor, added to low potassium dextran (LPD) used as a single-flush solution in normothermic ischemic animal models, bearing in mind the physiopathology of damage appearing during ischemic preservation and post-ischemic reperfusion injury.

2. Materials and methods

This study was accepted by Dicle University Health Sciences Research and Application Center (DUSAM), and was carried out at the center. Twenty-one New Zealand white rabbits were selected as the experimental subjects. These weighed an average of 3216 g (range 2950–3520 g). LPD solution, prepared by our biochemistry department, was used as a lung protection solution. One liter of LPD solution included 50 g of 40% dextran, 138 mmol Na⁺, 6 mmol K⁺, 142 mmol Cl⁻, 0.8 mmol SO₄, 0.91 g glucose, and HCl and NaOH in such a way as to give a pH value of 6, and distilled water was added to obtain a total of 1 l of solution. The prepared solution was kept at +10 °C until perfusion was applied.

2.1. Experimental protocol

The subjects were sedated with 35 mg/kg ketamine HCl (Ketalar® 50 mg/ml, Parke Davis, Istanbul, Turkey) and 5 mg/kg diazepam i.m. as premedication. Then 1000 units/kg heparin was given intravenously. For anesthesia, 25 mg/kg Na thiopental (Pentothal sodium® 1 g flk, Abbott, Italy) was applied intravenously. The trachea was then rapidly located in the neck with a cervical vertical incision and a tracheostomy was performed. The trachea was then intubated with a 3F intubation tube. The subjects were ventilated with 100% O₂ at a rate of 30 breaths/min at 10 ml/kg tidal volume by means of a mechanical ventilator. The carotid artery was located in the neck and a 24F angiocatheter was inserted to monitor blood pressure. Arterial blood pressure was sampled from all subjects (Stat profile M, Nova Biomedical, Waldham, MA). A median sternotomy was carried out together with a horizontal sternal incision, following

which the thymus was removed by thymectomy. In order to secure wide exposure of the left pulmonary hilus, the left 3rd, 4th and 5th ribs were excised almost totally and removed. After dividing the inferior pulmonary ligament, the lung was shifted toward the posterior with the help of a separator and the left pulmonary hilus was exposed. The left pulmonary artery and vein were found by dissection and suspended separately. After the pulmonary artery and vein had been clamped, a 24F angiocatheter was inserted in the left pulmonary artery, and following total expansion of the lung the left pulmonary artery was perfused with 40 cm water pressure with 50 mg/kg of protection solution. An average of 150 ml protection solution was given to every sample, and ventilation was continued with an inspired oxygen fraction of 1.0. Gradual whitening of the lung during infusion was observed. At the end of the perfusion, the left main bronchus was clamped and the lung was kept in ischemia for 120 min. Following ischemia, first the pulmonary vein, then the pulmonary artery and finally the pulmonary bronchus were declamped and reperfusion was carried out for a period of 90 min. The 21 samples were divided into three groups consisting of seven subjects.

1st or control group: lung protection solution was not used. After 120 min of ischemia 90 min of reperfusion was carried out.

2nd group: LPD solution was used as a lung protection solution. After 120 min of ischemia 90 min of reperfusion was carried out.

3rd group: 150 KIU/ml aprotinin (Trasyloi®, within 50 ml sterile isotonic solution, includes 70 mg aprotinin, equal to 500,000 KIU, Bayer, Germany) was added to the LPD solution as a lung protection solution. After 120 min of ischemia 90 min of reperfusion was carried out.

After tissue samples had been taken, the animals were sacrificed by intraperitoneal administration of 100 mg/kg Na-Pentothal at the end of the perfusion period. All experimental protocols were reviewed and approved by an institutional animal use committee. The animals received humane care in compliance with the 'Guide for the Care and Use of Laboratory Animals' formulated by the National Institute of Health (NIH publication No: 85-23, revised 1985).

2.2. Physiological parameters allotted

From the beginning of the experiment, systemic arterial pressure in the samples was recorded hourly and shown schematically via monitoring with the combination of a pressure line and cannula inserted in the carotid artery. To measure blood gases, blood samples were taken from the carotid artery at the beginning of the experiment as a baseline at the 15th and 60th minutes of reperfusion. Oxygen

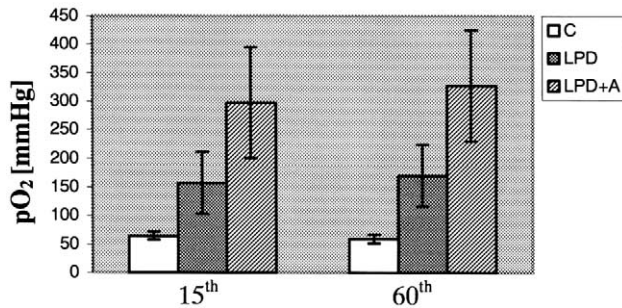


Fig. 1. Partial oxygen pressures (pO₂) detected in mmHg for all groups at the 15th and 60th minutes of reperfusion. pO₂ values were significantly higher in the LPD + A group versus LPD ($P < 0.001$) and C ($P < 0.001$) groups at the 15th and 60th minutes of reperfusion. A, aprotinin; C, control; LPD, low potassium dextran.

tension (pO₂) and alveolar-arterial oxygen difference (AaDO₂) were calculated from blood gas analysis.

2.3. Measurement of lipid peroxidation

At the end of reperfusion, the upper lobe of the left lung was excised to determine lipid peroxidation, and the tissues were kept in a deep freeze. The tissue samples were then homogenized in 1.15% KCl solution with a ultraturax homogenizer, after which 10% homogenized material was prepared. After this, 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid, and 1.5 ml of 8.1% thiobarbituric acid (TBA) solution were added to 0.2 ml of 10% homogenized material. After this mixture was expanded volumetrically to 4 ml with distilled water, it was kept in a boiling water bath for 1 h. Following these procedures, the mixture was cooled in a tap water bath, and 1 ml distilled water and 5 ml *N*-butanol/pyridine mixture were added to the mixture. This newly formed mixture was shaken vigorously. The butanol phase was separated by centrifugation at 4000 rev./min for 10 min, and the absorbance of colored complex was measured at 535 nm with a Shimadzu UV-1201 spectrophotometer. Lipid peroxidation was determined in the lung tissue homogenates with the TBA test as described by Ohkawa et al. [9], and the results were expressed in nanomoles of malondialdehyde (MDA), a last product of lipid peroxidation, per gram of wet tissue using a molar extinction coefficient of 1.56×10^5 at 535 nm for calculation.

2.4. Examination of bronchoalveolar lavage (BAL) fluid

The upper lobe of the left lung was excised and removed from the samples at the end of reperfusion. Then 5 cc of isotonic fluid was given to the upper lobe bronchus, and then taken back into the tube some time later. Thoma slides were used to measure the neutrophil percentage in the BAL fluid. This is a slide over which the material to be studied is spread in order to count the number of leukocytes under direct microscopy. The Thoma slide was closed with a cover slip and pressure was applied in order to attach it. BAL fluid was then inserted between the slide and the cover slip with a thin

pipette. The area was located in the Thoma measurement camera, and cells in four of 16 small squares in an area of 10×10 mm were counted. The figure obtained was then multiplied by 40 in order to determine the number of cells per mm³. In order to detect neutrophils, Methylene Blue was inserted between the slide and cover slip, thus coloring the cells, and the neutrophils in the area were counted and their percentage was calculated.

2.5. Morphological evaluation

The lower left lobe was excised from the samples and fixed with 10% neutral formalin. The preparations were sliced 3 μ m thick and each was stained with hematoxylin–eosin and examined under a light microscope with respect to alveolar hemorrhage and other parenchymal lesions. Microphotographs of them were then taken. Morphological examination relied on the alveolar hemorrhage grading system used by Higgins et al. [10], and the histological method of specimen examination under light microscope used by Egan et al. [11] (grade 0, no hemorrhage; grade 1, a few erythrocytes within the alveoli; grade 2, erythrocyte clusters that do not fill the alveoli completely; grade 3, erythrocyte clusters that fill the alveoli completely; normal, no lesion; mild, focal inflammation; moderate, perivascular peribronchial edema, vascular congestion, and inflammation; severe, intrapulmonary, interstitial edema together with severe vascular congestion and thrombosis).

2.6. Statistical evaluation

The results obtained in our study were calculated as an average \pm standard deviation. For statistical evaluation, the Statistica for Windows release 4-5[®] StatSoft Inc. 1993 program was used. As a statistical significance test, the Kruskal–Wallis ANOVA median test was employed.

3. Results

Two rabbits died from complications before the experiments. One of them died as a result of a massive hemorrhage due to pulmonary artery injury. The other rabbit died from cardiopulmonary arrest due to anesthesia before tracheostomy. These subjects were excluded from the study. Two out of 21 (9.5%) rabbits had complications during the procedure. One of the rabbits in the control group experienced a hemorrhage from the intercostal artery. This was halted by ligation and the procedure was continued. One rabbit in the LPD and aprotinin (LPD + A) group also suffered a bronchial rupture. The procedure on this animal continued once the rupture had been sutured.

3.1. pO₂

pO₂, the most important parameter of pulmonary function, was identified by blood gas analysis taken at the 15th and 60th minutes of reperfusion. The LPD + A group

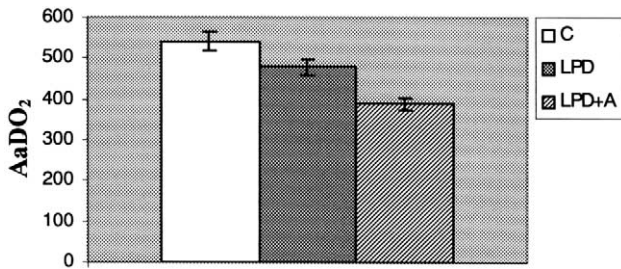


Fig. 2. AaDO₂ detected in mmHg for all groups at the 60th minute of reperfusion. The AaDO₂ values were significantly lower in the LPD + A group versus LPD ($P < 0.001$) and C groups ($P < 0.001$). A, aprotinin; C, control; LPD, low potassium dextran.

showed the highest levels of oxygenation at the 15th and 60th minutes of reperfusion (297 ± 76.7 and 327 ± 97.4 mmHg) versus the LPD (157 ± 20.6 and 170 ± 53.6 mmHg) ($P < 0.001$) and control (64 ± 8.4 and 59 ± 7.2 mmHg) groups ($P < 0.001$) (Fig. 1).

3.2. AaDO₂

Alveolar-arterial oxygen gradient, a good indicator of oxygen diffusion, was measured. Arterial blood gas analysis carried out at the 60th minute of reperfusion showed significant AaDO₂ differences between the three groups. The AaDO₂ values (389 ± 15 mmHg) of the LPD + A group were significantly lower than those of the control (542 ± 23 mmHg) ($P < 0.001$) and LPD (478 ± 19 mmHg) groups ($P < 0.001$) (Fig. 2).

3.3. MDA levels

MDA, the last product of lipid peroxidation, was measured in all subjects and no statistically significant difference was detected between the group in which only LPD solution was used (145.06 ± 9.5 nmol MDA/g) and the control group (147.30 ± 3.9 nmol MDA/g) ($P > 0.05$). MDA levels were significantly lower, however, in the LPD + A group (119.80 ± 5.3) than in the other two groups ($P < 0.005$) (Fig. 3).

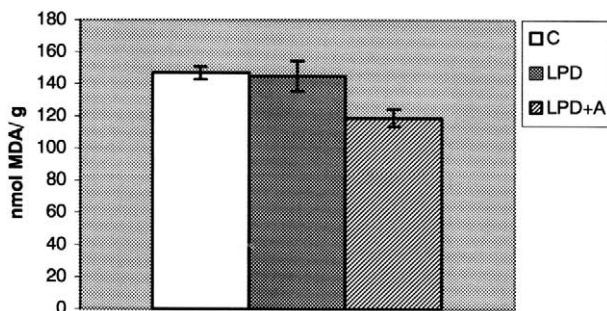


Fig. 3. MDA levels in lung tissue. MDA levels were significantly lower in the LPD + A group versus LPD ($P < 0.005$) and C groups ($P < 0.005$). A, aprotinin; C, control; LPD, low potassium dextran.

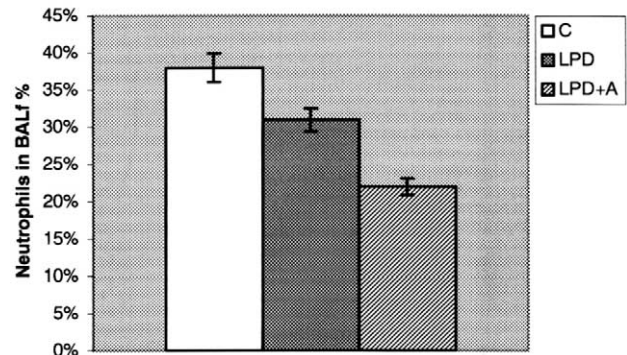


Fig. 4. Neutrophil percentage in bronchoalveolar lavage fluid (BALF) according to the groups. The LPD + A group had a significantly lower neutrophil percentage versus LPD ($P < 0.001$) and C ($P < 0.001$) groups. A, aprotinin; C, control; LPD, low potassium dextran.

3.4. Neutrophil studies

The percentage of neutrophils, the most important cell in inflammation of the lungs, in BAL fluid was calculated from values obtained from the Thoma camera. Those values were $22 \pm 2.4\%$ in the LPD + A group, but $31 \pm 6.1\%$ in the LPD group and $38 \pm 2.4\%$ in the control group. It was established that the LPD + A group had a significantly lower neutrophil percentage than the other two groups ($P < 0.001$) (Fig. 4).

3.5. Morphological analysis

Morphological evaluation, a parameter of lung preservation, was carried out with a light microscope. The percentages of pathological lesions and alveolar hemorrhage grades were established in all samples. The LPD + A group had a significantly lower percentage of pathological lesions and alveolar hemorrhage grade values than the LPD and control groups ($P < 0.005$) (Table 1).

Table 1
Morphological evaluation: percentage of pathological lesions and alveolar hemorrhage according to the groups^a

	C	LPD	LPD + A
<i>Alveolar hemorrhage (%)</i>			
Grade 0	–	–	15
Grade 1	–	14	57
Grade 2	42	71	28
Grade 3	58	15	–
<i>Pathologic lesions (%)</i>			
Normal	–	–	28
Mild	–	42	58
Moderate	57	42	14
Severe	43	16	–

^a C, control; LPD, low potassium dextran; A, aprotinin.

4. Discussion

Despite a great deal of research work performed on the subject of lung protection and the prevention of ischemia–reperfusion injury in lung transplantation, consensus has not been reached on the content of ideal perfusion, method of application, addition of suitable pharmacological agents and establishment of suitable conditions. In this study, in the light of the physiopathology of damage due to reperfusion injury, we used LPD as a single-flush solution in normothermic ischemic animal models, and demonstrated that the addition of aprotinin, a serine protease inhibitor, to the solution has a positive effect on lung protection and the prevention of reperfusion injury.

In virtually all clinical lung programs, the pulmonary artery flush temperature is 1–4 °C. Some investigators have shown that a more moderate degree of hypothermia (10 °C) results in superior lung function. This principle was demonstrated in an *in vitro* rabbit lung perfusion model [12] as well as in a standard model of canine left lung allotransplantation [13]. However, Mayer et al. [14] were unable to show a difference between storage at 4 °C versus 10 °C in canine left lung allografts. In our experimental studies we also performed lung perfusion at 10 °C, and sought to observe the effectiveness of aprotinin in ischemic-reperfusion injury under normothermic conditions. Since previous experimental studies usually focused on transplantation following preservation, studies on antioxidants are exclusively set up to prevent reperfusion injury. Many enzymes in catalytic cycles in an ischemic environment cause the formation of free radicals [1]. Of these, xanthine oxidase, which is the most studied enzyme, forms superoxide radicals while transforming from its xanthine dehydrogenase form to an oxidase form in ischemia [15]. Reductions in cell energy production and defective membrane functions cause intracellular calcium levels to rise due to ischemia. High intracellular Ca^{2+} concentrations activate protease, which transforms xanthine dehydrogenase into xanthine oxidase [16]. This transformation is completely preventable by protease inhibitors. Aprotinin, a protease enzyme inhibitor, inhibits the transformation of xanthine dehydrogenase into xanthine oxidase, thus preventing free oxygen radical formation [17].

MDA is a good indicator of free oxygen radical formation. MDA elevation shows increased lipid peroxidation due to the effects of free oxygen radicals [9]. The level of MDA in the LPD + A group was significantly lower than in the LPD and control groups ($P < 0.005$). However, there was no significant statistical difference between the LPD group and the control group ($P < 0.05$). Other researchers have also established that aprotinin reduces MDA levels.

The greater part of the specific reperfusion component of ischemic reperfusion injury is neutrophil-related organ injury. Due to the effect of endothelial-originated adhesion molecules, neutrophils attach themselves to endothelial tissue, and then secrete reactive oxygen and proteolytic

enzymes, resulting in functional and deep structural damage in lung parenchyma [4]. Aprotinin, which is a serine protease inhibitor, has an anti-proteolytic effect as a result of its ability to decrease intracellular cGMP levels and increase cAMP levels [7]. Sunamori et al. [18] showed that in myocardial protection and in the presence of aprotinin, cGMP levels were lower and cAMP levels were higher, and that lysosomal enzymes such as *N*-acetyl β -glucosaminidase were also lower. From these results they concluded that aprotinin has a lysosomal membrane stabilizing effect. In lung protection the most sensitive parameter of lung function is the preservation of oxygen capacity, the provision of better oxygen diffusion, and the reduction of edema formation [4]. It was shown that with the addition of aprotinin to organ protection solution in rat lungs, oxygenation and compliance increased, better oxygen diffusion occurred, and edema formation was reduced [7]. Our study also detected a significant increase in oxygen pressures 15 and 60 min after reperfusion and a significant decrease was detected in the alveolar-arterial oxygen gradient in the LPD + A group. Low AaDO₂ values were indicators of better oxygen diffusion, and this shows that the lungs are well protected. In morphological examination, when grading alveolar hemorrhage and histological examination of the specimens, recovery was observed in the LPD and LPD + A groups when compared to the control group. However, the only significant difference was between the LPD + A and control groups.

After cardiopulmonary bypass (CPB) lung injury occurred via neutrophils [19,20]. Neutrophils induce lung injury by releasing elastase and oxygen radicals. Aprotinin inhibits neutrophil elastase release [7,19,21]. In ischemia, TNF- α is first synthesized as a membrane precursor in damaged endothelial cells and macrophages, and with the effect of serine protease enzyme it is divided into mature TNF- α products. Via IL-8, TNF- α activates the neutrophils, stimulates their adhesion to the endothelial surface, and increases their superoxide production or phagocytosis actions with degranulation [19]. Aprotinin inhibits the serine protease enzyme that brings about the transformation of immature TNF into mature TNF- α . With the inhibition of TNF- α , the secretion of IL-8 decreases, reducing neutrophil accumulation, and as a result lung injury will be decreased [7,21]. In our study, the neutrophil percentage of the total cell count in the BAL fluid in post-reperfusion was considerably lower in the LPD + A group than in the LPD and control groups. Our results support the findings stated above.

In our gradation of alveolar hemorrhage and histological examination of the specimens during morphological examination, we established that the percentages of pathological lesions and alveolar hemorrhages were significantly lower in the LPD + A group in comparison to the other two groups. This is a result of aprotinin reducing neutrophil elastase secretion. Other researchers have reported similar findings [19].

Aprotinin has anti-inflammatory and hemostatic effects on the concentration of kallikrein inhibition. When kallikrein inhibition occurs, the production of direct precursors of the vasoactive peptide bradykinin is blocked. It prevents bradykinin secretion and blocks the production of high molecular weight kininogen [4]. There are studies reporting that reperfusion injury decreases with the suppression of bradykinin, which increases during ischemia and reperfusion, with the use of aprotinin [22]. In recent studies, it has been thought that nitric oxide plays a role in macrovascular injury at ischemia–reperfusion as a result of its reaction with the products of vascular and perivascular cells. It was detected that aprotinin inhibits cytokine-dependent nitric oxide synthetase and reduces the production of endogenous airway nitric oxide, as a result of which these organ injuries decrease [19,23].

In conclusion, because of its hemostatic features aprotinin reduces the need for transfusion in response to blood loss as a result of its widespread use in CPB. Moreover, in clinical use it prevents ischemia–reperfusion injury via multi-effect mechanisms and prevents primary graft dysfunction. Thus, the addition of aprotinin to lung protection solution in lung transplantation protocols will yield positive results.

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