

ORIGINAL PRE-CLINICAL SCIENCE

Treatment of the brain-dead lung donor with aprotinin and nitric oxide

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edema;
rat

BACKGROUND: It has been previously shown that donor treatment with aprotinin or inhaled nitric oxide reduces reperfusion injury after lung transplantation in animals. These studies used living donors with normal lungs. However, the main source of lungs for transplantation is brain-dead donors. Brain death causes systemic inflammatory response and lung injury, rendering the organ susceptible to reperfusion injury after transplantation. We hypothesized that treatment with aprotinin or inhaled nitric oxide after brain death would improve the donor inflammatory response and reduce lung reperfusion injury after transplantation.

METHODS: Brain death was induced in 24 rats by intracranial balloon inflation. Subsequently, the animals received intravenous aprotinin ($n = 8$), inhaled nitric oxide ($n = 7$), or no treatment ($n = 9$) for 5 hours. The lungs were retrieved and reperfused for 2 hours using recipient rats.

RESULTS: After brain death, oxygenation deteriorated earlier and significantly more in rats that received treatment, especially with nitric oxide. Treatment did not reduce the donor systemic inflammatory response as assessed by serum levels of proinflammatory cytokines. Oxygenation, airway pressure, pulmonary vascular resistance, lung water index and bronchoalveolar lavage cytokine levels were similar after reperfusion of grafts from all three groups of donors.

CONCLUSIONS: Donor treatment with aprotinin or inhaled nitric oxide does not improve lungs that have been injured by brain death.

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Brain death in the potential transplant donor frequently results in hypertensive crisis, followed by neurogenic hypotension.¹ We have previously established that these hemodynamic phenomena can cause an inflammatory response and lung injury.² We have also demonstrated that these mechanisms significantly contribute to primary graft failure after transplantation.³ Prevention of the hypertensive crisis in animal models is proven to avoid lung damage but

may not be practical in the clinical setting. However, we have shown that correction of the neurogenic hypotension with a vasoconstrictor such as noradrenaline or vasopressin is also helpful in reducing the donor inflammatory response.^{4,5} This treatment is feasible and should be considered advantageous in donor management.

Further attempts to reduce the donor inflammatory response are made after brain death, and administration of steroids is almost universal practice. In the present study, we investigate the effects of treatment with aprotinin and inhaled nitric oxide on the donor systemic inflammatory response as well as lung reperfusion injury. Both treatments have important anti-inflammatory properties observed mainly at the level of neutrophil-endothelial cell interactions.^{6–11} They have been previously

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studied in living donor animals and have shown to reduce lung injury after subsequent reperfusion.^{12–17} However, because most lung grafts come from brain-dead donors, the role of donor treatment with these agents should be evaluated in brain death models. To our knowledge, treatment with aprotinin or inhaled nitric oxide in lung injury after brain death or generally in the context of neurogenic pulmonary edema has not been evaluated before.

Methods

Rat lung transplantation was performed using outbred male Wistar rats (Charles River, UK), 24 donors (mean weight, 425 grams; range, 316–514 grams) and 24 recipients (mean weight, 450 grams; range, 332–502 grams). The procedures were performed humanely, according to the regulations of the Animals (Scientific Procedures) Act 1986, UK (license ref: PPL 60/2670).

All donors were anesthetized in a chamber with 5% isoflurane, intubated with a 14-gauge cannula, and ventilated with an Advanced Inspira Volume Control ventilator (Harvard Apparatus, UK) with a tidal volume of 10 ml/kg, frequency of 50 cycles/min, inspiration/expiration ratio of 1/2, and inspired oxygen fraction of 1.0. The rats were maintained with inhalation of 3% isoflurane. A tail vein was cannulated for fluid and drug administration. The left femoral artery was cannulated for blood pressure monitoring and blood sampling. Body temperature was maintained at 37.0°C using an electronic homeothermic blanket (Harvard Apparatus). All rats received an intravenous infusion a gelatin solution at 5 ml/kg/hour.

Experimental groups

The following 3 groups of donors were used.

1. Brain Death ($n = 9$). Brain death was induced by intracranial balloon inflation as previously described.² Brain death occurred almost immediately and was followed by cessation of respiratory effort, pupil dilatation, and disappearance of the corneal reflexes. Balloon inflation was also accompanied by a hypertensive crisis, which lasted for about 4 minutes and was followed by sustained neurogenic hypotension. Brain death was objectively confirmed by the disappearance of the brain stem auditory evoked response (BAER) after balloon inflation.¹⁸ The animals were monitored for 5 hours after brain death. Repeat testing showed no recovery of the BAER.
2. Aprotinin group ($n = 8$). Donor rats underwent the same procedures as the Brain Death group. However, the animals received aprotinin (Aprotinin Bovine Lung, BioChemika, Sigma-Aldrich Company Ltd, Dorset, UK) treatment throughout the 5-hour period. A loading dose of aprotinin (30,000 KIU/kg) was administered as an intravenous bolus within 5 minutes from balloon inflation. This was followed by continuous intravenous infusion of another 50,000 KIU/kg aprotinin over 5 hours.

3. Nitric Oxide (NO) group ($n = 7$). Donor rats underwent exactly the same procedures as the Brain Death group. However, the animals received continuous inhaled nitric oxide (20 ppm) treatment. Therapy was initiated within 5 minutes from balloon inflation and continued for 5 hours, including during lung retrieval.

Graft retrieval and transplantation

At the end of the 5-hour period, the lungs were retrieved en bloc with the heart, using a technique previously described.³ In brief, heparin (300 U/kg) was administered intravenously to the donor. After median sternotomy, the apex of the heart was excised across both ventricles. A silicone catheter (bore, 2.5 mm; wall, 0.5 mm) was inserted in the pulmonary artery through the right ventricle, and the lungs were perfused with 40 ml/kg of cold (4°C) low potassium dextran preservation solution (Perfadex, Vitrolife, Kungsbacka, Sweden) at 15 to 20 mm Hg pressure. The Perfadex solution was vented through the apical left ventricular opening.

After flush perfusion, ventilation was discontinued and the lungs were excised en bloc with the heart. The pulmonary artery was divided together with the aorta, near their origin and in toto with the sub-valvular right ventricular musculature. The left ventricular free wall was then fully opened with an incision made near the point where it meets the septum, and the mitral valve was closed with 7-0 Prolene (Ethicon, Somerville, NJ) suture. Pursestring sutures (6-0 Mersilk, Ethicon) were placed, 1 around the left atrial appendage and another surrounding the pulmonary artery. The lungs were inflated with 100% oxygen (8 ml/kg rat body weight), and the trachea was ligated. The block was placed in a closed container filled with Perfadex (4°C), and the container was stored in ice. After 3 hours of cold ischemic time, the lungs were reperfused using recipient rats, using a technique previously described.³ This model permits continuous monitoring of pulmonary artery flow and pressure, together with ventilatory indices. Recipients were monitored for 2 hours after graft reperfusion.

Sampling and analysis

In donors, arterial blood gases and the concentration of proinflammatory cytokines and chemokines in serum before and after brain death were measured with enzyme-linked immunosorbent assay (ELISA DuoSets, R&D Systems Europe, Abingdon, UK).

After graft reperfusion, pulmonary arterial and left atrial blood pressure, graft blood flow, and airway pressure were continuously recorded, and pulmonary vascular resistance was calculated: (mean pulmonary artery pressure – mean left atrial pressure)/flow. Blood gases were measured in blood sampled from the pulmonary artery and pulmonary vein stents after graft reperfusion. The recipient rats were killed 2 hours after reperfusion, and bronchoalveolar lavage (BAL) of the right lung was performed with 5 ml of normal saline for determination of proinflammatory cytokines and

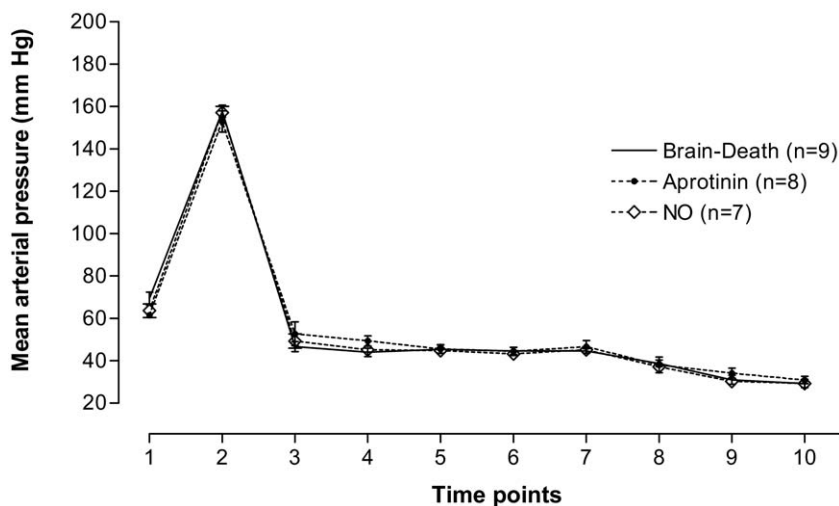


Figure 1 Donor mean arterial pressure (MAP) recorded at different time points in all experimental groups (mean \pm standard error of the mean). Brain death was induced by intracranial balloon inflation in rats. This was accompanied by a hypertensive crisis, followed by neurogenic hypotension. After induction of brain death, the rats received intravenous aprotinin ($n = 8$), inhaled nitric oxide (NO; $n = 7$), or no treatment ($n = 9$) and were monitored for 5 hours. Time points: (1) MAP before balloon inflation. (2) Peak MAP after balloon inflation. (3, 4, 5, 6, 7, 8, 9 and 10) 5, 15, 30, 60, 120, 180, 240, and 300 minutes after balloon inflation.

neutrophil concentration. The apex of the left lung was excised and weighed, dried in an oven at 80°C for 48 hours to constant weight, and the lung water index was calculated as (wet lung weight – dry lung weight)/wet lung weight. The rest of the left lung was snap frozen in liquid nitrogen and stored in -80°C .

Statistical analysis

Statistical analysis was performed using the SPSS 11.0 software (Chicago, IL). Data are reported as mean \pm standard error of the mean. Comparisons were based on pre-determined hypotheses. Multiple post hoc comparisons were avoided. When multiple measurements were taken over time, comparisons between 2 groups were made using

summary measures including the area under the curve, the highest recorded value, and the final recorded value. Comparisons between 2 independent groups of observations with normal distribution were performed using the Student's *t*-test. If the distribution was non-parametric, the Mann-Whitney *U*-test was used. Paired observations within a group were compared with the paired *t*-test (normal distribution) or the Wilcoxon signed ranks test (non-parametric distribution). Comparisons among 3 or more independent groups of observations with normal distribution were performed with one-way analysis of variance, or the Welch test, if the variances of the groups were unequal. The Kruskal-Wallis test was used for 3 or more groups of observations with non-parametric distribution. Differences were considered significant at the level $p \leq 0.05$.

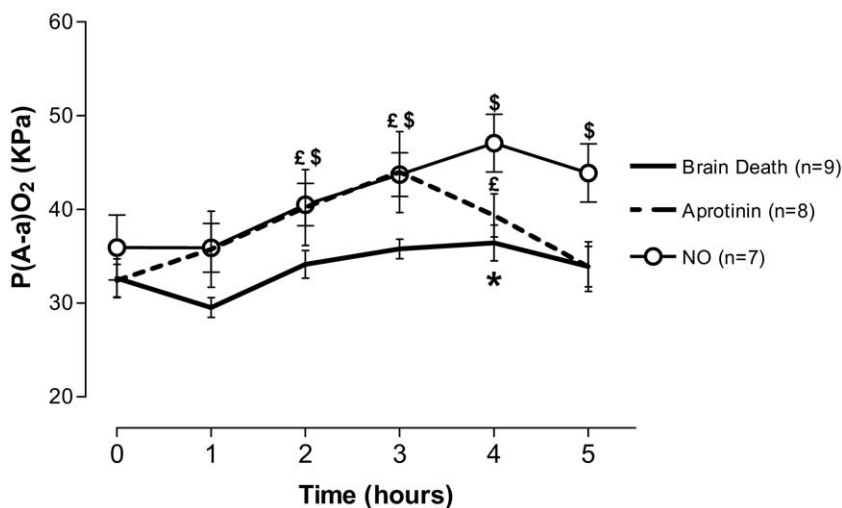


Figure 2 Changes in alveolar-arterial difference in partial oxygen pressure $P(A-a)O_2$ in brain-dead donor rats, which received aprotinin, inhaled nitric oxide (NO), or no treatment. Values at each time point are compared with baseline (time point 0: immediately before brain death) in each group. (*, £, \$) p = significant in Brain Death, Aprotinin, and NO groups, respectively.

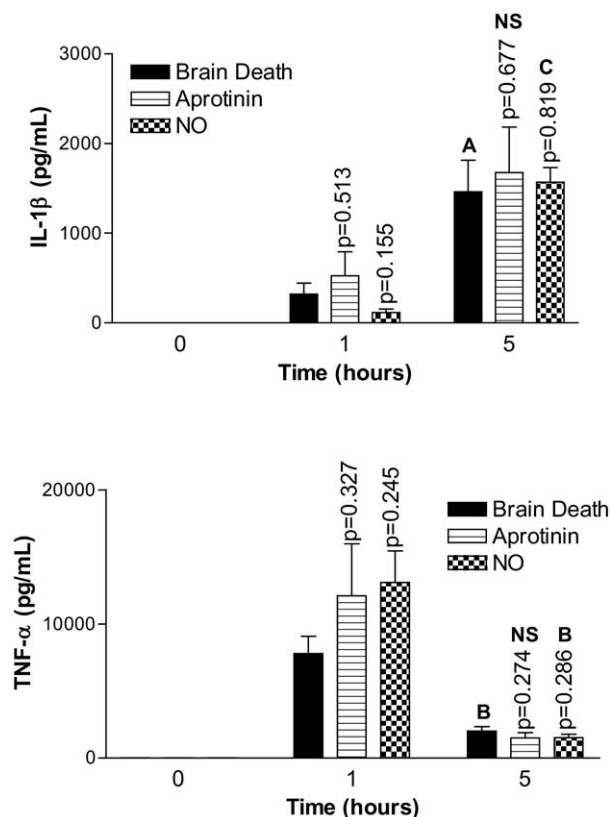


Figure 3 Serum levels of the cytokines interleukin-1 β (IL-1 β) and tumour necrosis factor- α (TNF- α) before (0), and 1 and 5 hours after brain death in donors. There was no difference in the serum cytokine levels between the untreated group (Brain Death) and the groups that received treatment with aprotinin (Aprotinin) or inhaled nitric oxide (NO). The *p*-values displayed are from comparisons with the Brain Death group. Also levels at 5 hours are compared with the levels at 1 hour in each group (NS, not significant; A, *p* < 0.05; B, *p* < 0.01; and C, *p* < 0.001).

Results

Hemodynamic changes

Baseline mean arterial pressure (MAP) was 68.0 ± 1.42 mm Hg and was similar in the donors of all experimental groups (*p* > 0.05; Figure 1).

After balloon inflation, the MAP increased acutely and rapidly to a peak of 158 ± 10.6 mm Hg (*p* < 0.001). Peak MAP was not different between the groups (*p* > 0.05). The hypertensive crisis lasted for about 4 minutes. MAP then decreased to a hypotensive plateau, which was not different between groups (mean, 42.5 ± 0.76 mm Hg; *p* = 0.281).

Donor oxygenation

Before brain death, there was no difference in the alveolar-arterial partial oxygen pressure gradient among the experimental groups (*p* = 0.1). Oxygenation deteriorated significantly after brain death in all groups; however, the deterioration occurred earlier and was worse in the Aprotinin and NO groups (Figure 2). At the end of the 5-hour

period, oxygenation recovered to baseline in Brain Death and Aprotinin groups and was similar in both (*p* = 0.1). In the NO group, oxygenation was still significantly inferior to baseline and the Brain Death group (*p* = 0.01).

Donor serum cytokines

Interleukin (IL)-1 β , tumour necrosis factor (TNF)- α , and cytokine-induced neutrophil chemoattractant (CINC)-3 were not detectable in serum before brain death. IL-1 β and CINC-3 increased progressively and significantly after brain death (Figures 3 and 4). TNF- α increased significantly within 1 hour after brain death and then decreased (Figure 3). Serum CINC-1 was detected at low concentrations before brain death and increased significantly and progressively (Figure 4). There was no difference in the serum cytokine levels between the untreated group and the groups that received treatment with aprotinin or nitric oxide.

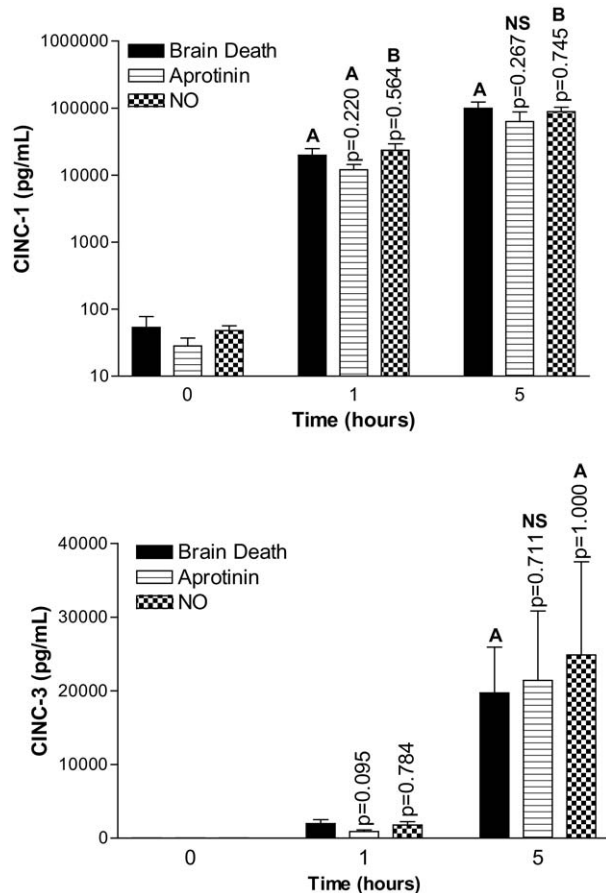


Figure 4 Serum levels of the chemokines cytokine-induced neutrophil chemoattractants -1 and -3 before (0), and 1 and 5 hours after brain death in donors. There was no difference in the serum chemokine levels between the untreated group (Brain Death) and the groups that received treatment with aprotinin (Aprotinin) or inhaled nitric oxide (NO). The *p*-values displayed are from comparisons with the Brain Death group. Also, levels at 5 hours are compared with the levels at 1 hour in each group (NS, not significant; A, *p* < 0.05; and B, *p* < 0.01).

Graft variables

After transplantation and reperfusion, there was no difference in the peak airway pressure, pulmonary vascular resistance, and alveolar-pulmonary venous partial oxygen pressure gradient among grafts harvested from the 3 donor groups (Figure 5).

At the end of the 2 hours of reperfusion, the lung water index was not different between the Brain Death group and the Aprotinin ($p = 0.352$) or the NO groups ($p = 0.896$; Figure 5). Similarly, BAL levels of the cytokines IL-1 β , TNF- α , CINC-1, and CINC-3 were not different between the groups (Figure 6). After reperfusion, the percentage of neutrophils in BAL leukocytes was significantly lower in

grafts from donors treated with aprotinin (Figure 7). However, the absolute neutrophil concentration was not different in any of the treatment groups compared with the Brain Death group (Figure 7).

Discussion

This is the first study, to our knowledge, to evaluate treatment of the brain-dead donor with aprotinin or inhaled nitric oxide. Treatment with either agent did not reduce the systemic inflammatory response in the donor, as indicated by the serum cytokine levels. Deterioration in oxygenation

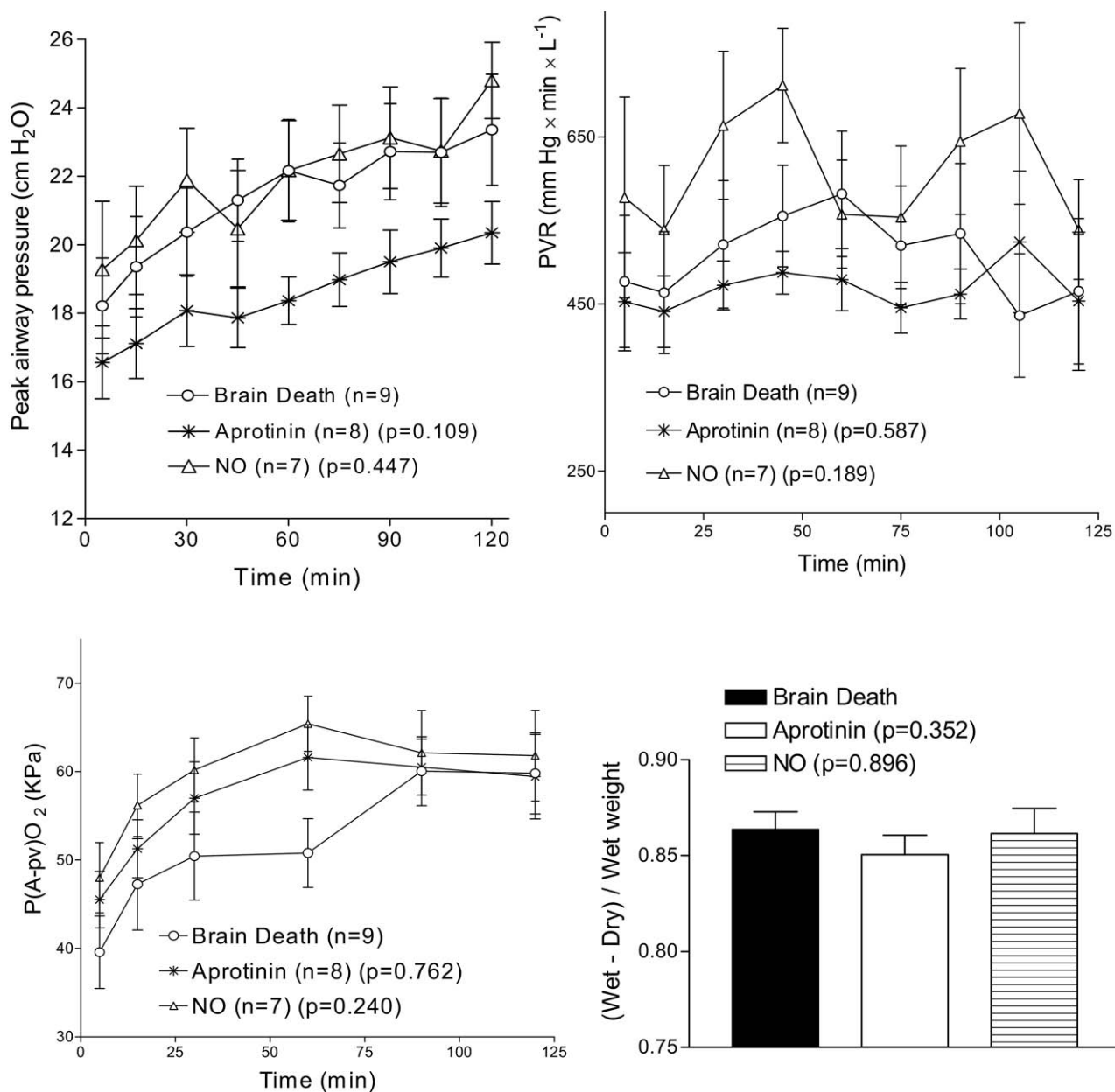


Figure 5 Graft peak airway pressure, pulmonary vascular resistance (PVR), alveolar-pulmonary venous partial oxygen pressure gradient P(A-pv)O₂ and lung water index after transplantation. The Brain Death group is compared with the Aprotinin (p -value in brackets) and the NO group (p -value in brackets). Donor treatment with aprotinin or inhaled nitric oxide made no difference in the performance of the graft and the amount of pulmonary edema after transplantation.

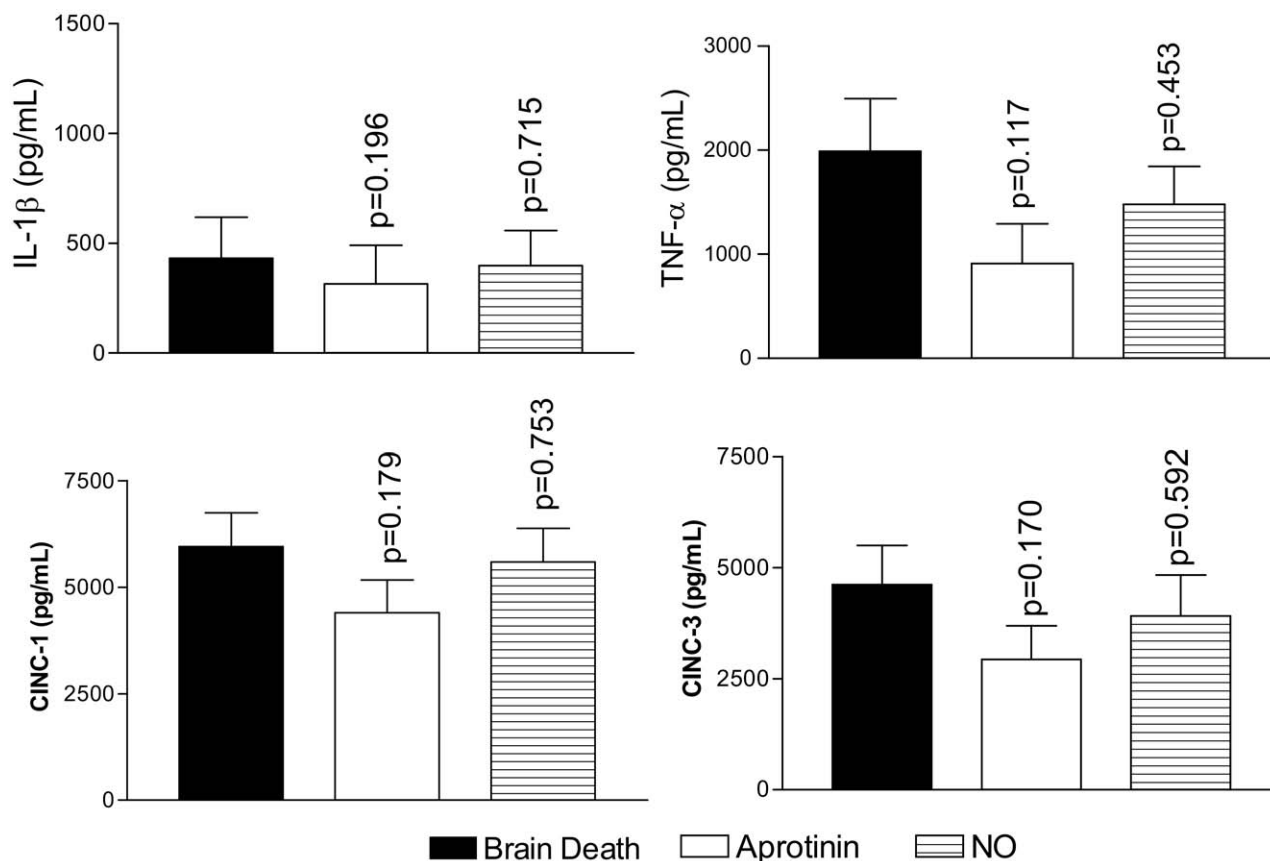


Figure 6 Graft bronchoalveolar lavage cytokine levels 2 hours after transplantation. The Brain Death group is compared with the Aprotinin and the NO group. Donor treatment with aprotinin or inhaled nitric oxide made no difference in the proinflammatory cytokine levels in the lung after transplantation.

occurred earlier and was more severe in rats treated with either agent than in untreated donors. However, oxygenation in the Aprotinin group did return to levels similar to the untreated group at 5 hours.

The exact causes behind these findings cannot be determined from the data. It is possible that both treatments further increased the early permeability alterations caused by the sympathetic discharge, but oxygenation improved later in the Aprotinin group due to an anti-inflammatory effect on the lung. This can only be a speculation, however, because neither permeability nor inflammatory markers were measured in the donor lungs before reperfusion. However, it can safely be concluded that neither treatment succeeded in reducing the measured systemic inflammatory markers or improving lung function in the donors. Moreover, donor treatment with either agent did not affect graft outcome after reperfusion. Grafts from the Brain Death, Aprotinin, and NO groups showed similar airway pressure, pulmonary vascular resistance, oxygenation, water index, and BAL cytokine levels.

A difference was found in the BAL neutrophils, when expressed as a percentage of leukocytes, which was significantly lower in the Aprotinin group; however, the absolute concentration of neutrophils in the BAL fluid was not different between the groups. The percentage of neutrophils may be the preferred measure when there are significant differences in the amount of pulmonary edema, which can

cause dilution and reduce the absolute concentration of neutrophils. In our study, there was no significant difference in the lung water index between groups, and therefore, the absolute concentration of neutrophils should be a satisfactory measure. Aprotinin has been previously shown to reduce primary adhesion and activation-dependent binding of neutrophils to the endothelium and subsequent migration.⁶⁻⁸ Our study did not provide clear evidence that administration of the drug to the donor may decrease neutrophil migration in the lung after reperfusion. Any such effect was weak and did not improve early graft function. This series of experiments did not specifically study whether it could improve the outcome by ameliorating the damage to the lung and shorten the course of primary graft dysfunction.

Inhaled NO treatment, to our knowledge, has not been studied in the context of donor brain death. There is only one case report of administration of inhaled NO to a pediatric organ donor with beneficial effects.¹⁹ The present study suggests that such treatment has no benefit and may even have detrimental effects. However, NO was administered in donor rats ventilated with 100% oxygen. NO, apart from its protective effects, can also have toxic effects on the lung. The toxic effects are partly due to formation of NO₂ and ONOO⁻ when it reacts with oxygen or O₂⁻. Although the net effect of nitric oxide may be protective due to its scavenging actions on more hazardous reactive oxygen spe-

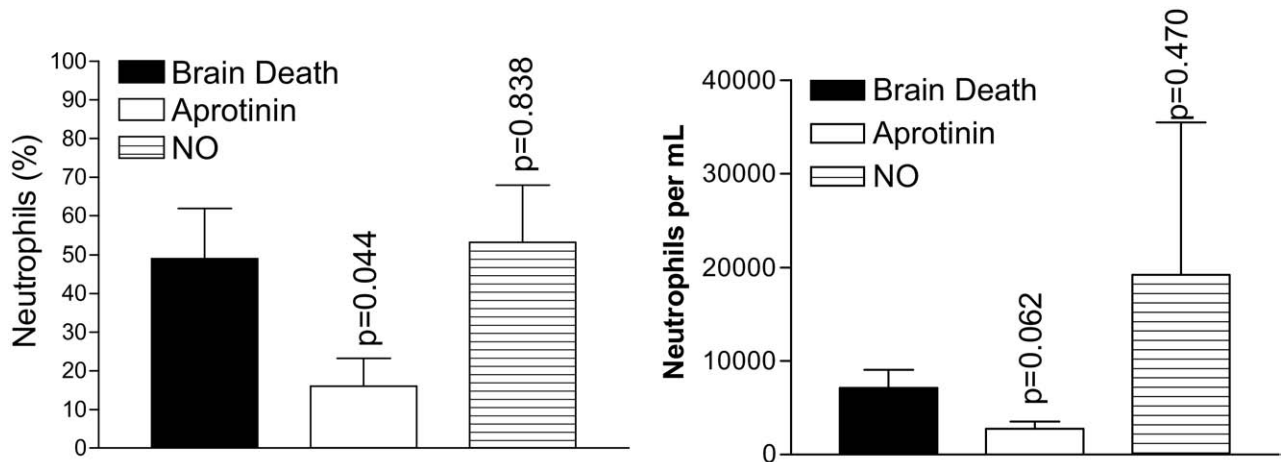


Figure 7 Graft bronchoalveolar lavage neutrophils 2 hours after transplantation. Brain Death group is compared with the Aprotinin and the NO group. Although the percentage of neutrophils in the graft bronchoalveolar lavage leukocytes was lower in the Aprotinin group than in the Brain Death group, the absolute concentration of neutrophils in the lavage was not different. Treatment with inhaled nitric oxide made no difference.

cies (H_2O_2 , alkyl-peroxides and O_2^-), toxicity may prevail at high oxygen concentrations.^{20,21}

Previous studies of donor treatment with inhaled nitric oxide showed a beneficial effect on reperfusion injury,^{15–17} apart from one study by Hausen et al²² using the transplant model described here. That study showed inferior outcome with nitric oxide. However, there are two fundamental differences among the previous studies and the one described here. First, this study applied the treatment to brain-dead donors, whereas previously, nitric oxide was administered to living donors. Second, the present study treated the donors for 5 hours, starting immediately after brain death and continued during retrieval. Previous studies exposed the donors to a relatively brief period of inhaled nitric oxide treatment immediately before retrieval. So although the present study aimed to treat brain death-induced lung injury, the others targeted the process of ischemia-reperfusion alone. Similar was the use of aprotinin in previous studies, where the agent was added to the preservation solution.

In a previous human randomized trial conducted by our group, transplanted lungs from brain-dead donors were treated with inhaled nitric oxide at the time of reperfusion. We found no clinical benefit and a trend toward increased proinflammatory cytokines in the treated lungs.²³ This is further evidence that nitric oxide may provide no benefit in lung transplantation from brain-dead donors irrespective of the time of administration.

Using the present model, we have previously published data demonstrating the difference between lung grafts from brain-dead and sham-operated donors. Specifically, we have shown that brain death results in donor systemic inflammatory response, lung injury, and post-transplantation reperfusion injury. The differences between the present Brain Death group and a group of sham experiments were shown in those previously published studies.^{2,3} For this reason, those data are not described here.

The proinflammatory cytokines IL-1 β , TNF- α , and rat chemokines CINC-1 and CINC-3 measured in the present

experiments were chosen due to their important role in the development of lung injury.^{24–29} Our group was the first to describe the effects of rat brain death in the serum and lung levels of the rat chemokines CINC-1 and CINC-3.^{2,3}

We conclude that treatment with aprotinin or inhaled nitric oxide after brain death does not reduce the systemic inflammatory response and may adversely affect lung function in the donor, without any significant benefit on graft outcome after transplantation. These findings stress further the overwhelming impact of the hypertensive phase on donor inflammation and lung reperfusion injury. They also highlight the fact that living donor experiments may not be sufficient for investigating new treatments. Studies should be carried out in organs from brain-dead donors before the beneficial effects of such treatments are granted.

Disclosure statement

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