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Phosphodiesterase type 4 inhibitor prevents acute lung injury induced by cardiopulmonary bypass in a rat model☆

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Abstract

Objectives: Cardiopulmonary bypass (CPB) induces systemic inflammatory response with neutrophil activation and subsequent lung dysfunction. Rolipram, a selective phosphodiesterase type 4 inhibitor, blocks the decrease in levels of cyclic adenosine monophosphate associated with neutrophil activation. Here, we tested the protective effect of rolipram on CPB-induced lung injury in the rat. **Methods**: Rats were divided into three groups: control (C), rolipram (R) and sham (S). In the C and R groups, animals underwent CPB at a flow rate of 60 ml/kg per min for 60 min followed by another 60-min observation, whereas the S group rats were sustained for 120 min only with median sternotomy and the placement of cannulae for CPB. Rolipram (40 μg/kg per min) was administered to the R group rats by continuous intravenous infusion from 10 min before the establishment of CPB to the end of the experiment. **Results**: The R and S groups showed significantly higher mean arterial oxygen pressure and lower mean lung wet-to-dry weight ratio compared with those observed in the C group (R: 489 ± 44 or S: 527 ± 55 vs. C: 287 ± 185, and R: 5.0 ± 0.4 or S: 4.7 ± 0.3 vs. C: 5.9 ± 0.5, respectively; $P < 0.01$). Although CD11b expression levels on circulating neutrophils in the C group doubled after CPB, those in the R and S groups remained almost the same ($P = 0.0008$). Intrapulmonary tumor necrosis factor-α concentrations (pg/μg protein) in the C group tended to be higher than those observed in the R and S groups (R: 5.2 ± 2.1, S: 5.0 ± 2.1 and C: 8.9 ± 5.4; R vs. C: $P = 0.09$ and S vs. C: $P = 0.08$). Pathological study of lungs revealed that more alveolar hemorrhage and neutrophil accumulation were observed in the C group compared to the R and S groups. **Conclusions**: These results suggest that rolipram prevents acute lung injury via the inhibition of neutrophil activation during and after CPB in this setting of a rat model.

Keywords: Phosphodiesterase type 4 inhibitor; Cardiopulmonary bypass; Neutrophil activation; Rat

1. Introduction

Cardiopulmonary bypass (CPB) is known to induce the systemic inflammatory response with activation of neutrophils and other blood components such as complement and platelets [1,2]. These responses injure various organs and increase morbidity and mortality after cardiac surgery using CPB. Lung is one of the vulnerable organs for the attack of activated neutrophils. The incidence rate and mortality of acute respiratory distress syndrome in patients undergoing CPB was reported to be 0.5–1.7 and 50–91.6%, respectively [3]. To protect vital organs from this harmful response induced by CPB, several methods have been applied. For example, use of a filter that physically removes neutrophils from circulating blood [4], administration of specific substances to prevent the activation of complement [5] and antibodies for adhesion molecules [6].

Cyclic adenosine monophosphate (cAMP) has suppressant effects on neutrophil function such as superoxide anion production, adhesion molecule expression, tumor necrosis

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factor-α (TNF-α) production and F-actin assembly. Phosphodiesterase (PDE) inactivates cAMP by hydrolytically cleaving the 3′-phosphoester bond to form the corresponding inactive 5′-nucleotide monophosphate products, and type 4 is the predominant isoform of PDE in leukocytes. Therefore, the inhibition of PDE type 4 (PDEIV) increases the concentration of intracellular cAMP to attenuate neutrophil excitation [7].

Sato et al. [8] has recently demonstrated that a selective PDEIV inhibitor, rolipram, suppresses certain kinds of neutrophil activation induced by simulated extracorporeal circulation. The results indicate the possibility of using the PDEIV inhibitor to protect patients who undergo cardiovascular surgery with CPB from the detrimental systemic inflammatory response. In the current study, we tested the in vivo effect of PDEIV inhibition using a rat CPB model.

2. Materials and methods

2.1. Animals and reagents

Male Sprague–Dawley rats (14-week-old, 420–500 g) were purchased from a licensed vendor (Japan SLC, Inc., Shizuoka, Japan). They were housed in an air-conditioned room with free access to food and water at all time. All animal maintenance protocols were in compliance with the European Convention on Animal Care and approved by the Institutional Animal Care and Use Committee at the National Cardiovascular Center Research Institute, Osaka, Japan.

Rolipram (Sigma-Aldrich Japan K.K., Tokyo, Japan) was reconstituted to 10 mg/ml with dimethyl sulfoxide, and then diluted in saline with 5% bovine serum albumin (BSA) for use.

2.2. Surgical procedure

Rats were divided into three groups: control (C), rolipram (R), and sham (S) ($n = 6$ in each group). Each animal was anesthetized intraperitoneally with 50 mg/body of pentobarbital sodium and placed in the supine position. After insertion of a 14-gauge cannula into the trachea, mechanical ventilation was performed during the entire experiment by a ventilator (Model SN-480-7, Shinano Seisakusho Co., Ltd, Tokyo, Japan) with 8 ml/kg of tidal volume, 70 breaths/min of respiratory rate, 100% of inspired oxygen fraction and 3 cmH_2O of end-expiratory pressure. A 22-gauge cannula was inserted into the right jugular vein to infuse saline with 5% BSA at 3 ml/h and a 24-gauge cannula placed in the left femoral artery to monitor arterial blood pressure. For CPB, a 22-gauge cannula for arterial return was introduced into the right carotid artery and a 14-gauge cannula with side holes for venous drainage was positioned in the right atrium via the right appendage following median sternotomy. The CPB circuit comprising a membranous oxygenator (Senko Medical Co., Ltd, Osaka, Japan), tubing line with reservoir (Senko Medical Co., Ltd) and roller pump (NBM-1000, Senko Medical Co., Ltd) was primed by 9 ml of saline with 5% BSA just before use. After 200 units/kg of heparin was intravenously administered, CPB was established and maintained at 60 ml/kg per min of flow rate for 60 min except in the S group rats, which were submitted to the 120-min observation without CPB after insertion of all cannulae and then sacrificed. In C and R groups, the left bronchus, pulmonary artery and pulmonary vein were clamped all together at the left pulmonary hilum by a vascular clip and the tidal ventilation volume was decreased to 6 ml/kg during CPB. Furthermore, the R group rats received 40 μg/kg per min of rolipram intravenously from 10 min before the establishment of CPB to the end of the experiment. When CPB was terminated, the pulmonary clamp was released and the tidal volume was returned to the initial level (8 ml/kg). The rat was observed for another 60 min after CPB before sacrifice. Arterial blood pressure and peak airway pressure (P-AwP) were continuously monitored. Rectal temperature was maintained between 34 and 36 °C throughout the experiment. Arterial blood samples were obtained three times (before CPB, end of CPB and end of the experiment) for the measurement of arterial oxygen pressure (PO_2) and blood hemoglobin levels. The left lung was excised immediately after being sacrificed. Rats in which the hemoglobin level declined to less than 6 g/dl at any point were excluded from the study. There were four animals eliminated in total, including two in the C group and two in the R group.

2.3. Wet-to-dry weight ratio of the left lung

The left lung was divided into three parts. The superior third was used for the calculation of wet-to-dry weight (W/D) ratio. The lung block was weighed before and after desiccation for 72 h in a dry oven at 70 °C.

2.4. Expression of adhesion molecules on circulating neutrophils

The expression of CD11b and CD62L on neutrophils surface were analyzed by flow cytometry before and at the end of CPB. Fifty microliters of whole blood taken at each time point was incubated with 0.5 μl of phycoerythrin conjugated anti-rat CD11b (Serotec Ltd, Oxford, England) and 1 μl of fluoresein isothiocyanate conjugated anti-rat CD62L (BD Bioscience Pharmingen, San Diego, CA) at room temperature for 30 min. Erythrocytes were then destroyed by incubation with 1 ml of lysing solution (BD Bioscience Pharmingen) for 10 min. After washing twice by phosphate-buffered saline (PBS) and centrifuge at 500 × g for 5 min, neutrophils were fixed with 500 μl of CellFIX (Becton Dickinson, San Jose, CA). Leukocyte populations were identified by forward and side scatter signals and gated

appropriately. Percentage positivity and mean channel fluorescence intensity were determined by 2000 cells of gated neutrophils. CD11b and CD62L expressions were evaluated as percentage change in GEO mean from the values before CPB.

2.5. Measurement of TNF-α in lung tissue

The middle third of the lung sample, which was immersed into the liquid nitrogen and stored at −80 °C, was utilized for the measurement of TNF-α concentration in lung tissue. The sample was homogenized in 0.5 ml of PBS containing protease inhibitor, sonicated, and then centrifuged at 10 000 rpm for 20 min at 4 °C. The amount of TNF-α in the supernatant was assayed using a specific ELISA kit for rat TNF-α (BioSource International, Inc., Camarillo, CA). The optical density of each well was read at 450 nm by a MAXline™ microplate reader (Molecular Devices Corp., Sunnyvale, CA). Finally, the concentration of TNF-α was corrected by the protein content that was determined by the Bradford method [9].

2.6. Pathological study

The inferior third of the lung sample was fixed with 10% formalin, embedded in paraffin, cut into 4 μm section and stained with hematoxylin and eosin (H/E) for pathological examination.

2.7. Statistical analysis

All data are expressed as mean ± standard deviation (SD). Comparison among groups was performed using analysis of variance (ANOVA). Fisher PLSD post hoc test was used for analysis between groups. All statistical analyses were performed using StatView for Macintosh 5.0 (Abacus Concepts, Berkeley, CA). Significance was set at $P < 0.05$.

3. Results

As for mean body weight, body temperature, hemoglobin value and PO_2, there were no significant differences among the groups before CPB.

3.1. P-AwP, PO_2 and W/D ratio of the left lung

Aeration and blood flow of the left lung of each rat was blocked by hilar clamping during CPB to simulate the pulmonary condition when total extracorporeal circulation is performed, and observed for 60 min after declamping. Although mean P-AwP in the C group was slightly higher than that observed in the R and S groups 60 min after CPB, the difference among the groups was not statistically significant (R: 10.3 ± 0.8 and S: 10.4 ± 1.0 vs. C: 12.8 ± 4.1 cmH_2O, $P = 0.18$) (Fig. 1A). PO_2 in the C group decreased significantly to 287 ± 185 mmHg at the end of the experiment, whereas the R group remained high PO_2 as well as the S group (C: vs. R: 489 ± 44 or S: 527 ± 55 mmHg, $P < 0.01$) (Fig. 1B). The C group also showed significantly higher W/D ratio than the other two groups. The value in the R group was almost same as that in

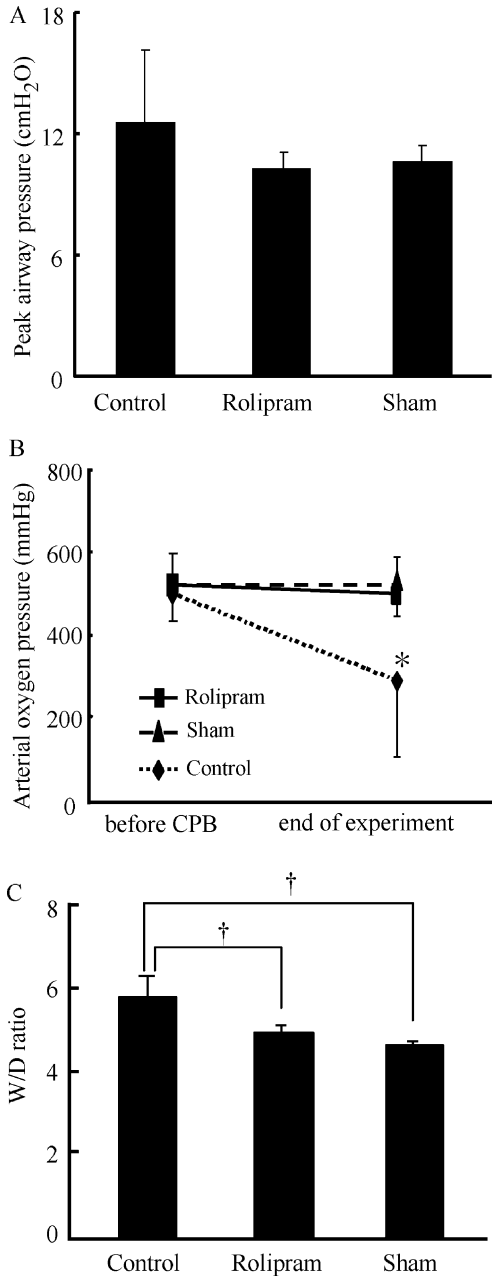

Fig. 1. (A) Peak airway pressure at the end of experiment. There are no significant differences among the groups, although the C group shows a relatively higher value than the others. Results are expressed as mean ± SD. (B) Arterial oxygen pressure (PaO_2) before CPB and at the end of experiment. The C group shows a significantly lower value than the others at the end of experiment. Results are expressed as mean ± SD. *$P < 0.01$ vs. the R or S group. (C) W/D ratio of the left lung. The C group shows a significantly higher value than the others. Results are expressed as mean ± SD. †$P < 0.01$.

the S group. (C: 5.9 ± 0.5 vs. R: 5.0 ± 0.4 or S: 4.7 ± 0.3, $P < 0.01$) (Fig. 1C).

3.2. Change in CD11b and CD62L expressions on neutrophils

As indexes of neutrophil activation induced by CPB, change in the expression of both surface adhesion molecules of CD11b and CD62L were analyzed. In the C group, the signal intensity of CD11b increased to more than the double after CPB while the R and S remained at relatively lower values (R: 112 ± 34 or S: 147 ± 56 vs. C: 242 ± 50% of pre-CPB level, $P < 0.01$) (Fig. 2A). As for CD62L, all groups showed mildly increased expression levels at the termination of CPB and there was no significant difference among the groups (Fig. 2B).

3.3. Tissue TNF-α concentration of the left lung

TNF-α is one of the major proinflammatory cytokines secreted from neutrophils. After CPB, tissue TNF-α concentration of the left lung in the R group was comparable to that in the S group. The C group showed higher TNF-α level than either the R or S group, although statistical significance was not recognized among the groups (R: 5.2 ± 2.1, S: 5.0 ± 2.1, C: 8.9 ± 5.4 pg/μg protein, R vs. C; $P = 0.09$ and S vs. C; $P = 0.08$) (Fig. 3).

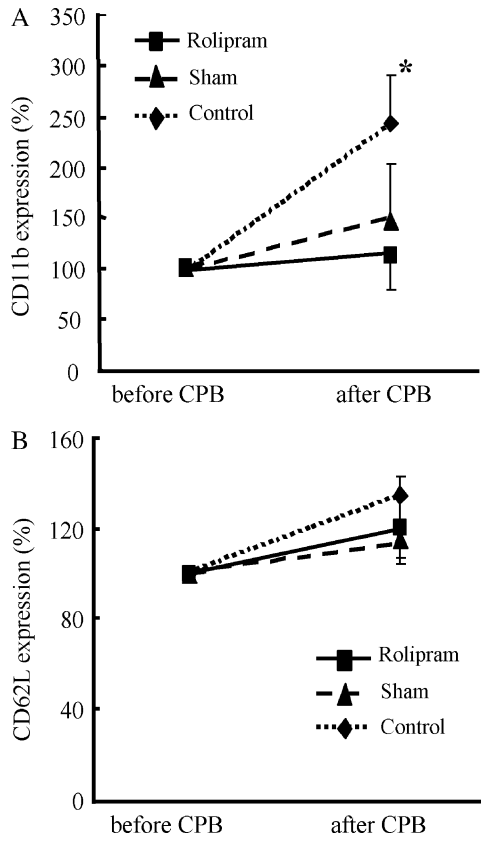

Fig. 2. (A) CD11b expression on circulating neutrophils after CPB. Percentage change in GEO mean from the value of before CPB was calculated. The C group shows a significantly higher value than the others. *$P < 0.05$ vs. the R or S group. Results are expressed as mean ± SD. (B) CD62L expression on circulating neutrophils. Percentage change in GEO mean from the value of before CPB was calculated. There are no significant differences among the groups. Results are expressed as mean ± SD.

3.4. Pathological findings of the left lung

H/E stained sections of the left lung were utilized for pathological examination. In the C group, there were more alveolar hemorrhage, interstitial edema and neutrophil accumulation compared to the R or S group (Fig. 4).

4. Discussion

CPB-induced acute lung injury is a life-threatening complication after major cardiac surgery and is thought to be associated with neutrophil activation. The present study uses a rat model to ascertain whether rolipram inhibits intracellular stimulatory signaling of neutrophils aroused by CPB and thereby protects the lung.

To evaluate neutrophil excitation, change in the signal intensity of adhesion molecules, CD11b and CD62L, on their surfaces were analyzed, and the concentration of TNF-α in the left lung was also measured. CD11b is a major integrin adhesion molecule that contributes to the tight adherence between neutrophils and endothelial cells [10]. This binding occurs in a sequential manner after the rolling process and plays a very important role for the further activation of neutrophils. CD62L is a member of the selectin family that is highly expressed on inactivated neutrophils in bone marrow [11] and participates in the initial weak adherence between neutrophils and endothelial cells [12]. Activated neutrophils promote rapid shedding of CD62L as the inflammatory response advances [13,14]. Therefore, when neutrophils are becoming activated, the expression of CD62L is decreased and that of CD11b is usually upregulated [12,15]. The fact that the expression of CD11b in the R group remained

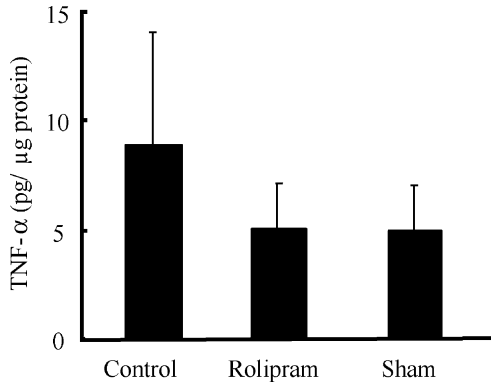

Fig. 3. Tissue TNF-α concentration in the left lung. There are no significant differences among the groups, although the C group shows a relatively higher value than the others. Results are expressed as mean ± SD.

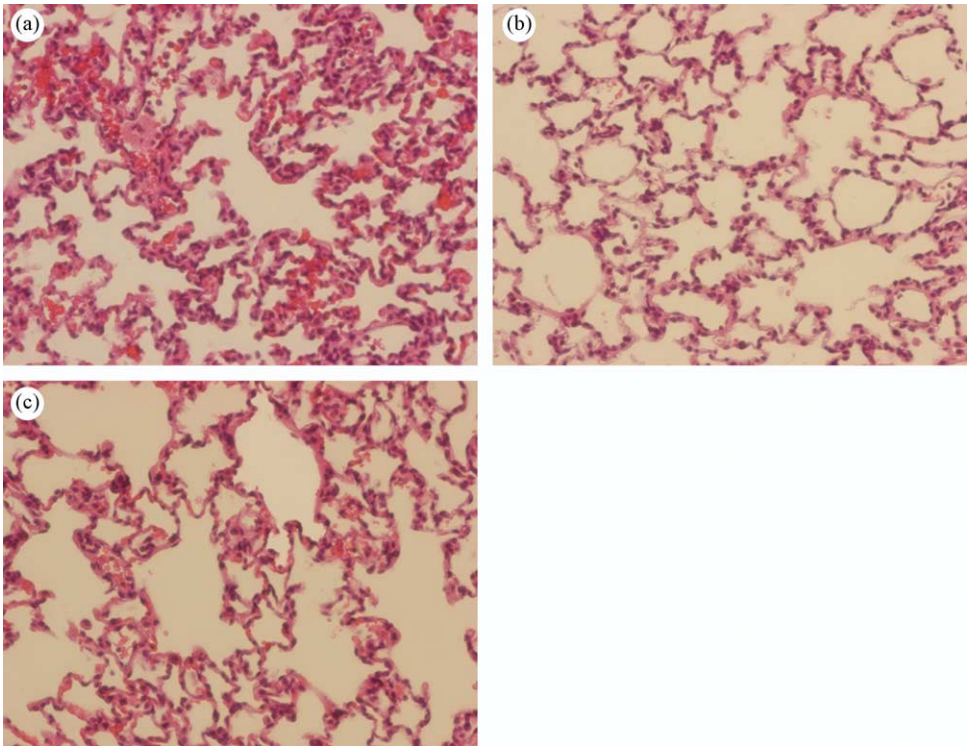

Fig. 4. Microphotograph of the left lung. The left lung section in each group was stained by hematoxylin and eosin. Only the C group lung shows a massive alveolar hemorrhage and severe neutrophil accumulation. Original magnification: 200 × . (a) The C group, (b) the R group, and (c) the S group.

almost unchanged after CPB while that in the C group increased markedly, suggests that rolipram attenuates the neutrophil activation induced by CPB. In contrast, all groups showed slightly increased expression levels of CD62L at the end of CPB. A possible reason why the R group did not show decreased expression of CD62L could be that it represents not only progressive shedding in the rolling process but also an accelerated migration of CD62L-abundant neutrophils from bone marrow. TNF-α is a representative proinflammatory cytokine that promotes tissue injury by stimulating neutrophils and other immune cells [16], and endothelial cells as well [17]. Regarding the TNF-α concentration of the left lung, the R group had lower levels than the C group and was comparable to the S group. The results imply that rolipram inhibits neutrophil accumulation in the left lung and/or TNF-α production in neutrophils. It is also consistent with previous studies [18,19].

Lung integrity was verified by PO_2, P-AwP, W/D ratio and pathological study. In this study, the left lung of the rat was left without ventilation and pulmonary blood supply during CPB to mimic the conditions of total extracorporeal circulation. The lung with 60-min of collapse and ischemia, and subsequent reperfusion should become more susceptible to activated neutrophils. Indeed, the left lung of the C group showed increased W/D ratio indicating the occurrence of pulmonary edema, marked alveolar hemorrhage and neutrophils infiltration in pathological examination. Decreased PO_2 observed in the C group also seems to appear to be due to the injured left lung. Interestingly, there was no statistical difference in P-AwP among the groups, although the C group showed slightly higher values. The right lung of the rat, which is by far larger than the left, might compensate for the elevation of airway pressure occurred in the latter. Rats in the R group, similar to the S group, conserved pulmonary integrity shown as higher PO_2, lower W/D ratio and minimal microscopic structural changes. Rolipram would contribute to the protection of lung during and after CPB through neutrophil inactivation. Furthermore, alveolar macrophages in lung also might be regulated by rolipram, because PDEIV is a major subtype among them.

In conclusion, a selective PDEIV inhibitor, rolipram, prevented acute lung injury associated with CPB via the inhibition of neutrophil activation in this rat model. This strategy might be a prophylactic option when major cardiac surgery with CPB is performed.

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Appendix A. Conference discussion

Dr D. Chambers (*London, UK*): Very interesting study. I wonder whether you measured cyclic AMP levels in your system?

Dr Hamamoto: In this study we did not measure the level of cyclic AMP.

Dr U. Lonn (*Uppsala, Sweden*): Are you using any other phosphodiesterase inhibitors in your routine practice, or are your anesthesiologists?

Dr Hamamoto: I didn't try the other type of phosphodiesterase type 4 in this experiment or clinical setting.

Dr Lonn: No, but in the clinical setting, are they using a lot of these substances in your clinical practice? I mean, if you want to extrapolate these results over to the clinical practice later on which I suppose you will do.

Dr Hamamoto: But we used a rat model. Our next step is, I think, a more larger animal study and then use the humans or clinical setting.

But rolipram was formerly studied as an antidepressant drug in 1984. And at the time, rolipram was taken orally and a major side effect was nausea and vomiting. And now, this drug was not used for the antidepressant drug. On the other hand, the other type of PDE type 4 inhibitor is commercially available in this fall for the treatment of asthma or COPD. The effectiveness of the drug results in bronchodilation.

Dr A. Corno (*Lausanne, Switzerland*): I also have a great admiration for your technical expertise to put these small animals on bypass. And I have a curiosity. You have applied your system to healthy rats. Don't you think then you should now, instead of move to the large animals, apply the same protocol to rats with some pulmonary problem? For instance, if you take rats exposed to 2, 3 weeks of chronic hypoxia, as we do for other reasons, you can reproduce the same protocol with animals with pulmonary hypertension and elevated pulmonary vascular resistance, and see if you obtain the same results. I would like to have your opinion on this possibility.

Dr Hamamoto: As for the effect of rolipram on these sick lungs, it may works more or less. But, I do not have any data about it so far. To use this drug for another animal, for example, a larger animal, I think, the same protocol will be tried at first. But the cardiopulmonary bypass circuit or the method or duration of the cardiopulmonary bypass time will be changed in the second step experiments.