Serial Epithelial Lining Fluid Collection Using Bronchoscopic Microsampling in a Canine Lung Transplantation Model

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Objectives: As a less invasive alternative to bronchoalveolar lavage (BAL), bronchoscopic microsampling (BMS) was developed to identify molecules present in the epithelial lining fluid (ELF) of the distal airways. Here, we evaluated the utility of BMS for serial collection of ELF in a canine lung transplant model.
Methods: ELF was collected hourly by BMS up to 5 hours after reperfusion in a canine left lung transplant model. Tumor necrosis factor (TNF)-α levels in ELF were measured using an enzyme-linked immunosorbent assay and were compared with those in BAL fluid.
Results: Serial collection of ELF by BMS in graft lungs was possible without adverse effects. However, the partial pressure of oxygen was markedly decreased because of ischemia-reperfusion lung injury. Probe ELF absorption ranged from 1-18 μL. TNF-α expression was significantly elevated and detected for 5 hours after reperfusion, whereas it was very low in the sham-operated group ($P < 0.05$). TNF-α concentration in BAL fluid was less than one-hundredth of that in ELF.
Conclusions: BMS was safe and effective for serial ELF collection in grafted lungs. Temporal changes in TNF-α corresponded with ischemia-reperfusion lung injury. This is the first study to adopt BMS to elucidate pulmonary function after lung transplantation.

Key words: Bronchoscopic microsampling, Epithelial lining fluid, Ischemia-reperfusion injury, Lung transplantation, Tumor necrosis factor-α

INTRODUCTION

Analysis of the epithelial lining fluid (ELF) of the distal airways provides a multitude of data useful for determining lung condition. In particular, measurement of biological markers associated with pulmonary disorders such as cytokines and chemokines has helped elucidate the pathophysiology underlying various lung disorders [1, 2]. Bronchoalveolar lavage (BAL) is a standard ELF collection technique. However, because BAL uses a substantial amount of saline, it could cause or aggravate existing lung injuries. Therefore, it is not suitable for serial analysis of lung health in patients with severe conditions. Another disadvantage of BAL is that dilution of the ELF by saline can be problematic for detection or quantification of biological markers or bioactive agents. As an alternative, the minimally invasive bronchoscopic microsampling (BMS) technique was developed. BMS uses probes made from urethane that absorbs ELF in the distal airways [3, 4]. BMS has been used previously to determine the pathophysiology underlying acute respiratory distress syndrome, acute or chronic lung injury, infectious diseases, and malignant lung tumors [5-8].

Lung transplantation is a treatment option for end-stage lung disease. However, despite considerable recent progress in lung transplant management, the outcome is often unsatisfactory because of ischemia-reperfusion lung injury, acute or chronic tissue rejection, or infection [9-12]. A transbronchial lung biopsy (TBLB) is the gold standard used to assess the condition of grafted lungs or to diagnose disorders [13]. However, because invasive TBLBs can cause bleeding, pneumothorax, or pneumonia, clinicians often hesitate to implement them, especially during the acute phase in severe conditions or after lung transplantation.

The purpose of the present experiment was to establish and validate the BMS method for serial ELF collection using a canine lung transplant model, which we hope might lay the foundation for the future use of BMS in lung transplant patients. Time-dependent changes in the ELF concentration of tumor necrosis factor (TNF)-α, a proinflammatory cytokine associated with ischemia-reperfusion lung injury [14, 15], were measured.

MATERIALS AND METHODS

The study protocol was approved by the Animal Experimentation Committee of Tokai University, and all animals used in this study received humane care in compliance with the Animal Experimental Guidelines.

Animals

Ten beagle dogs (CLEA Japan, Inc., Tokyo, Japan) weighing 10-12 kg were used. Four weight-matched pairs were assigned to the transplant groups and two
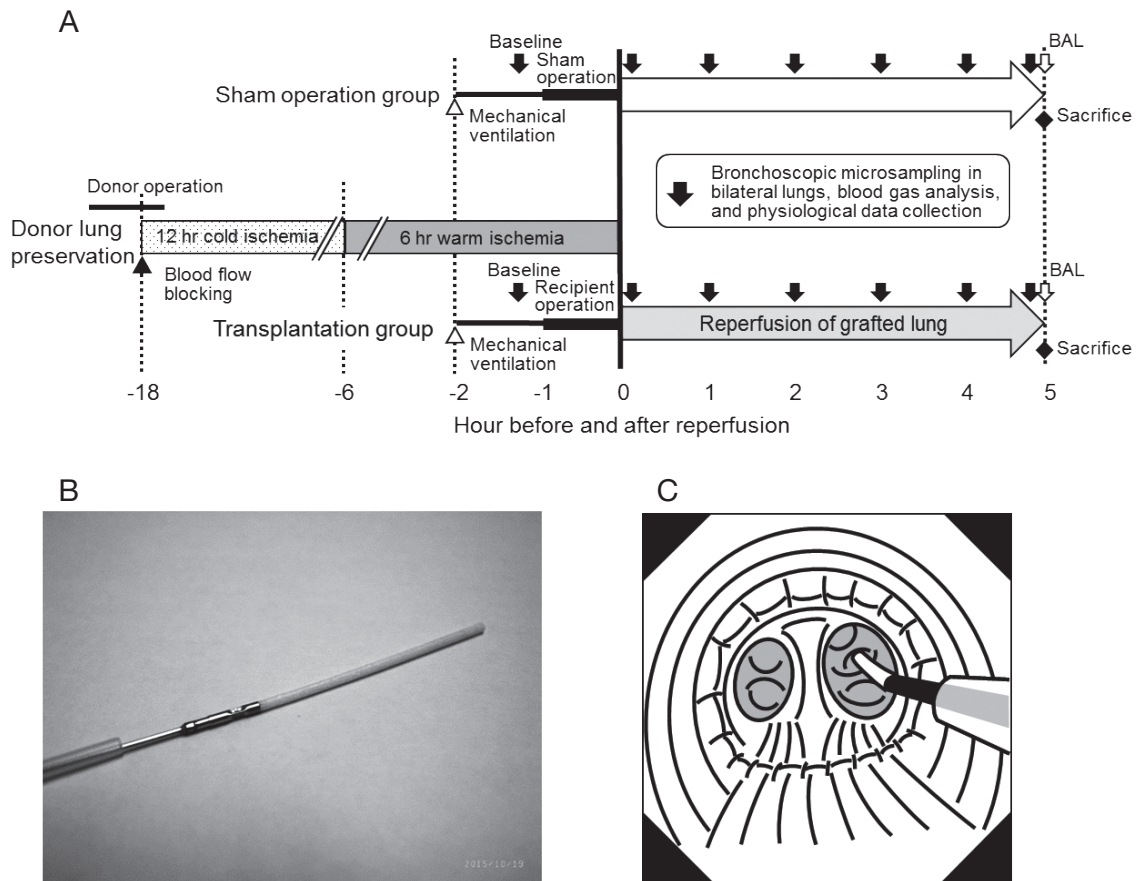
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Fig. 1 Experimental protocol and the bronchoscopic microsampling (BMS) probe.
(A) Schematized representation of the time course of interventions and samplings in the present protocol.
(B) The BMS probe has a polyethylene outer sheath and a 1.8-mm diameter and 30-mm long inner probe with a polyurethane adsorptive tip at the distal extremity, which is capable of absorbing up to 20 µl of liquid.
(C) A schema of the bronchoscopic view during BMS collection of epithelial lining fluid (ELF) in a grafted lung. A BMS probe is advanced through the forceps channel of the bronchoscope into the distal airway until slight resistance is perceived. The inner probe is left in place for 10 seconds to absorb ELF before its withdrawal through the outer sheath.

dogs comprised the sham-operated control group. The schematized experimental protocol is shown in Fig. 1A.

Lung procurement

Donor dogs were anesthetized with an intramuscular injection of medetomidine 20 µg/kg plus midazolam 0.3 mg/kg. Dogs were intubated using an endotracheal tube (8-mm inner diameter) and ventilated mechanically using a ventilator with a 25-mL/kg tidal volume at a rate of 15 cycles per minute, a positive end-expiratory pressure (PEEP) of 5.0 cm H_2O, and a fraction of inspired oxygen (FiO_2) of 1.0. Anesthesia was maintained by 3–5% sevoflurane inhalation and intravenous injection of rocuronium bromide (0.4 mg/kg/hour). A median sternotomy was performed. After intravenous administration of 5000 IU heparin, the pulmonary artery was cannulated and flushed with 1000 mL of ET-Kyoto solution (Otsuka Pharmaceutical Factory Inc., Tokushima, Japan), an extracellular preservation solution used for clinical lung transplantation [16], at a temperature of 4°C and pressure of 30 cm H_2O, with continued ventilation. The donor heart and lungs were harvested *en bloc*. The lungs were semi-inflated to within 20 cm H_2O with 100% oxygen, then soaked in the preservation solution and stored at 4°C for 12 hours, and at 20°C thereafter for 6 hours to enhance ischemia-reperfusion lung injury (Fig. 1A).

Recipient surgery

Recipient dogs were anesthetized, maintained, and ventilated in the same manner as the donors. Peak inspiratory pressure was monitored via the tracheal tube. For each recipient, a Swan-Ganz catheter (F7; Edwards Lifesciences, Irvine, CA) was placed in the left main pulmonary artery, accessed from the right femoral vein, to measure pulmonary arterial pressure (PAP). A femoral arterial line was inserted to measure arterial pressure and for arterial blood gas analysis. Baseline physiological indices and blood gas analysis data were obtained before the sham or transplant procedure. Following a left pneumonectomy via a left posterolateral thoracotomy, the left atrium, the left main bronchus, and the left pulmonary artery were anastomosed, and left lung transplantation was conducted [17]. The implantation time was set at 60 minutes. Blood flow and ventilation to the transplanted lungs were reestablished after 18 hours of ischemia. Thereafter, the chest was closed roughly, and general anesthesia was maintained

for 5 hours. During this time, physiological data such as systolic arterial blood pressure, heart rate, mean PAP, and peak airway pressure were recorded, and arterial blood samples were collected for blood gasses analysis. Sham-operated dogs underwent dissection of left bronchi, pulmonary arteries and veins alone, under the same setting as the transplant group.

Serial ELF collection using BMS

The BMS probe (Olympus Corporation, Tokyo, Japan) consists of a polyethylene outer sheath and a 1.8-mm diameter and 30-mm long inner probe, with a polyurethane adsorptive tip at the distal extremity, which is capable of absorbing up to 20 µL of liquid (Fig. 1B). An airway connector (Bodai Bronch-Safe, Double Swivel for Bronchoscopy; Sontek Medical Inc., Hingham, MA) was attached to the endotracheal tube forming an air-tight seal around the bronchoscope for continuous ventilation and PEEP maintenance during BMS. After introducing the flexible fiber optic bronchoscope (6-mm diameter) via an endotracheal tube, ELF was collected by introducing the BMS probe through the forceps channel, and advancing it into the distal airway until slight resistance was met (Fig. 1C). When introduced to the main bronchus, the broncho-scope confronted the inlet of the caudal lobe bronchus. Therefore, the probe insertion into the segmental or subsegmental bronchi of the caudal lobes would have a minimal risk of damaging the anastomotic region of the main bronchus in the transplanted lungs. For the ELF collection in the contralateral lungs and sham-operated dogs, the BMS probes were similarly introduced to the caudal lobes. The inner probe was left in place for 10 seconds before it was withdrawn via the outer sheath. When BMS was performed on a grafted lung, the bronchoscope was placed in the proximal side of the anastomosis of the left main bronchus and the probes were introduced into the distal bronchus taking care not to irritate the bronchial anastomosis. After withdrawal, each wet probe tip was weighed, the ELF collected was extracted with 1 mL of saline, and each probe was dried and weighed again. The amount of ELF collected was calculated as the difference between wet and dry weights. The extracted sample was spun at 3000 rpm for 15 minutes, and the supernatant was stored at −80°C. ELF was collected from the grafted left caudal lung and the right contralateral lung just after reperfusion and every hour thereafter until 5 hours after the start of reperfusion. Before left pneumonectomy, ELF samples were collected from the recipients' original left and the right lungs to obtain the baseline data (Fig. 1A).

BAL fluid collection

Animals were sacrificed, and the bilateral lungs were removed 5 hours after the start of reperfusion. BAL samples were collected from the transplanted left cranial lobes and the contralateral right cranial lobes after intrabronchial injection of 50 mL of saline. The BAL fluid was spun at 3000 rpm for 15 minutes, and the supernatant was stored at −80°C.

TNF-α measurements

The concentrations of canine TNF-α in ELF and BAL fluid were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine® ELISA; R & D Systems, Minneapolis, MN). Assuming that 1 mL = 1000 mg, the weights of the BMS samples were converted to volumes and the concentration of TNF-α in ELF (in pg/mL) was calculated as the concentration of TNF-α in 1 mL of saline × 1 mg/wet weight (mg) − dry weight (mg).

Histopathology and immunohistochemistry

The transplanted left caudal lobes and the contralateral right caudal lobes were used for histopathology. They were inflated with 10% buffered formalin at a pressure of 10 cm H_2O for fixation, then embedded in paraffin, and cut into 4-µm sections, and stained with hematoxylin and eosin. The paraffin-embedded organs were also processed for immunohistochemical analysis with an anti-TNF-α antibody (ab6671; 1 : 100 dilution; Abcam, Inc., Cambridge, MA). Tissue was blocked with 10% serum for 20 minutes at room temperature, and antigen retrieval was performed by heating the tissue sections in citrate buffer (pH 6.0). Samples were incubated with primary antibody for 30 minutes at room temperature. A biotin-conjugated goat anti-rabbit IgG polyclonal antibody (1 : 2000 dilution) was used as the secondary antibody.

Statistical analyses

The data are presented as means ± standard deviations. Physiological data, blood gas data, and TNF-α concentrations were compared between groups using repeated-measures analysis of variance (ANOVA). Changes from baseline or differences of ELF volume collected using BMS between groups were compared using the Student's paired or unpaired *t*-test. A *P* value < 0.05 was considered statistically significant. The data were analyzed using StatView-J 5.0 (Abacus Concepts Inc., Berkeley, CA).

RESULTS

Physiological indexes during serial implementation of BMS

Systolic arterial blood pressure, heart rate, mean PAP, and peak airway pressure were monitored serially and recorded after the start of reperfusion and throughout the serial ELF collection by BMS (Fig. 2). In the lung transplant group, systolic arterial pressure decreased, whereas heart rate increased after the start of reperfusion (*P* < 0.05; Fig. 2A & B). According to repeated-measures ANOVA, after reperfusion, there were significant differences in the systolic arterial pressure and the heart rate between the groups (both *P* < 0.005, Fig. 2A & B). However, both stabilized during the serial ELF collection. The mean PAP did not change significantly except at the 3-hour time point in the transplant group (Fig. 2C). Peak airway pressure was stable throughout the experimental period (Fig. 2D).

Blood gas analysis after lung reperfusion and throughout serial ELF collection using BMS
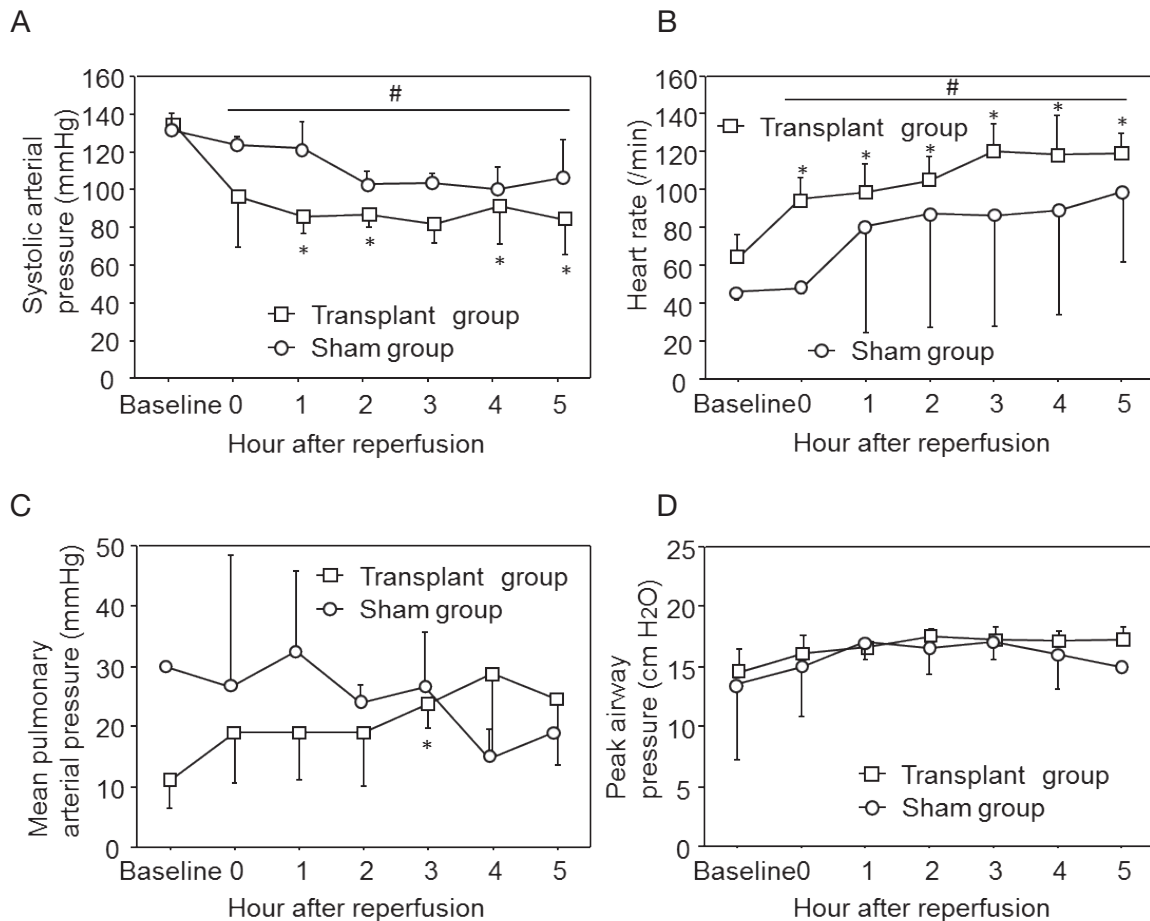
In the sham-operated group, PaO_2 was stable at

Fig. 2 Physiological measurements.
(A) Systolic arterial blood pressure, (B) heart rate, (C) mean pulmonary arterial pressure, and (D) peak airway pressure after surgery and reperfusion, and throughout the serial epithelial lining fluid (ELF) collection using bronchoscopic microsampling (BMS) in the lung transplant group (open square) and sham-operated group (open circle). Each point and bar represents the mean ± SD. *$P < 0.05$, compared with the baseline. #$P < 0.05$, differences between groups by repeated-measure analysis of variance.

around 400 mmHg throughout the experiment using the ventilator settings, FiO_2 1.0 and PEEP 5-cm H_2O. PaO_2 of the lung transplant group was significantly lowered at 3 to 5 hours after reperfusion ($P < 0.05$; Fig. 3A). There was a significant difference in the PaO_2 between the groups after reperfusion compared by repeated-measures ANOVA ($P = 0.0001$; Fig. 3A). In the transplant group, $PaCO_2$ tended to be higher, although the differences were not significant between groups (Fig. 3B). There was a significant difference in the pH between the groups after reperfusion compared by repeated-measures ANOVA ($P < 0.01$; Fig. 3C).

ELF volume collected using BMS

The frequency of distribution of the ELF volume collected is shown in Fig. 4A. The mean ELF volume was $6.0 ± 4.9$ µL (range, 1–18 µL). The ELF volume collected from the lung transplant group tended to be greater than that collected in the sham-operated group. Moreover, the ELF volumes collected from the grafted left lungs were significantly greater compared with those collected from the sham-operated left lungs ($P < 0.05$; Fig. 4B).

TNF-α concentration comparisons between ELF and BAL fluid

In the transplanted group, ELF TNF-α concentrations collected from the grafted and contralateral lungs were measured, while those obtained in the sham-operated group were mostly lower than measurement ranges (Fig. 5A & B). However, in the grafted lung, compared with baseline, there was a significant elevation in the ELF TNF-α concentration at 4 hours after the start of reperfusion. Furthermore, throughout the experiment, there were significant differences in the ELF TNF-α concentrations between the grafted left and the sham-operated left lungs, and between the contralateral lungs in each group ($P < 0.05$, Fig. 5A & B). By contrast, after 18 hours of ischemia and 5 hours of reperfusion, the BAL TNF-α concentrations were very low and were similar to those in the sham-operated group (Table).

Histopathology and immunohistochemical staining of TNF-α in grafted lungs

On histologic examination, severe alveolar hemorrhage, infiltration of inflammatory cells, and interstitial thickening were observed in the grafted lungs of
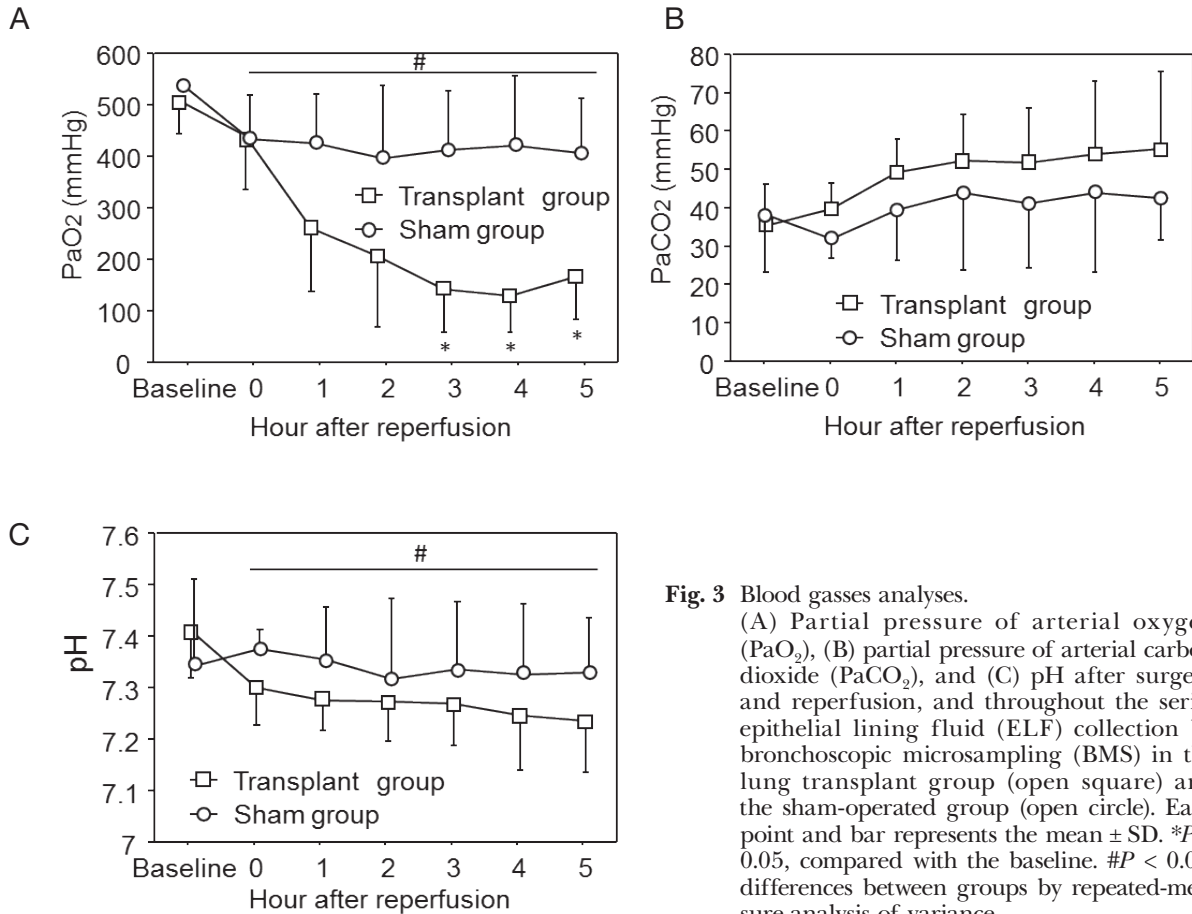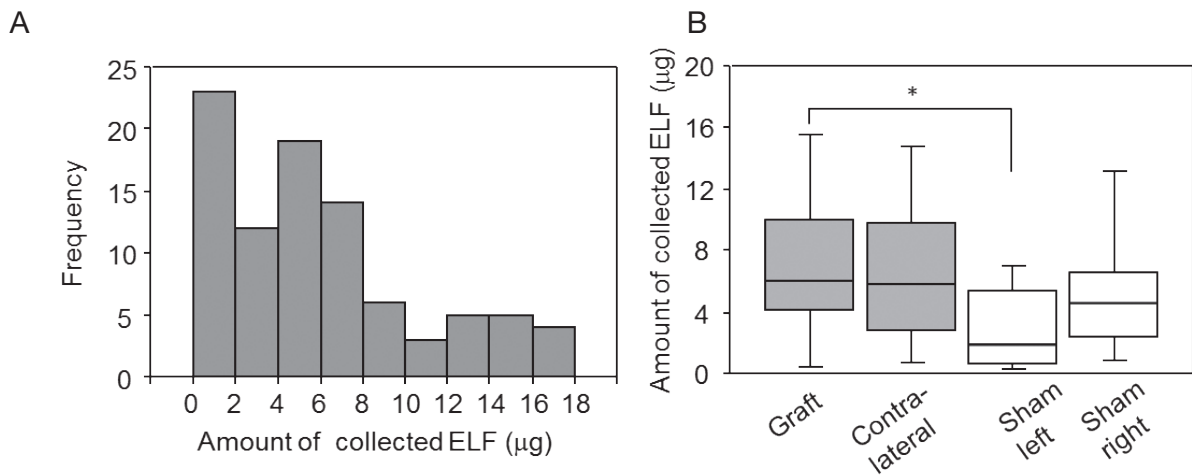
A

B

C

Fig. 3 Blood gasses analyses.
(A) Partial pressure of arterial oxygen (PaO_2), (B) partial pressure of arterial carbon dioxide ($PaCO_2$), and (C) pH after surgery and reperfusion, and throughout the serial epithelial lining fluid (ELF) collection by bronchoscopic microsampling (BMS) in the lung transplant group (open square) and the sham-operated group (open circle). Each point and bar represents the mean ± SD. *P < 0.05, compared with the baseline. #P < 0.05, differences between groups by repeated-measure analysis of variance.

A

B

Fig. 4 Volume of distribution histograms.
(A) Histogram showing the volume distribution of epithelial lining fluid (ELF) sampled using bronchoscopic microsampling (BMS), regardless of the group, in a canine lung transplant model. (B) Box and whisker plot showing the volume of ELF sampled by BMS technique on a canine lung transplant model according to the group, the transplanted and the contralateral lungs of the lung transplant group (gray boxes) and the left and right lungs of the sham-operated group (white boxes). Data are presented as mean ± SD. *P < 0.05, differences between groups, Students t-test.
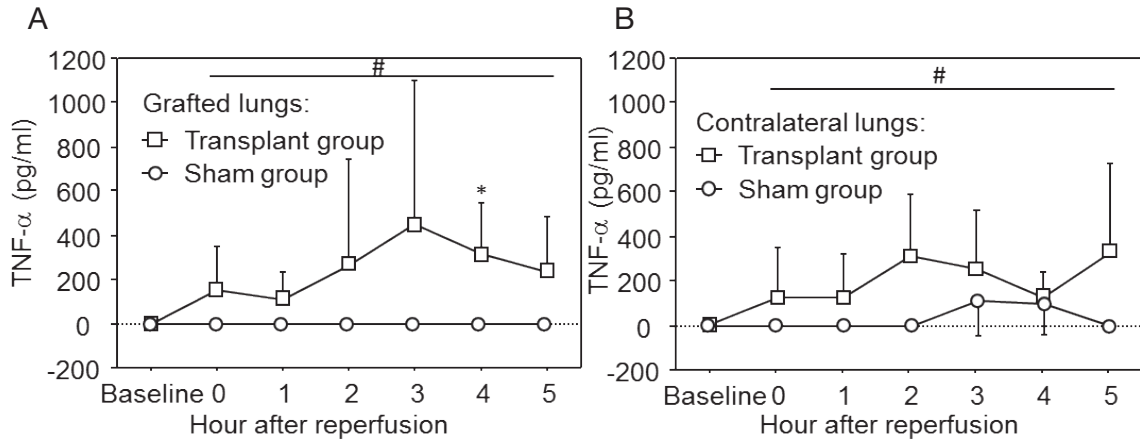
Fig. 5 Temporal changes in tumor necrosis factor (TNF)-α concentrations in epithelial lining fluid (ELF) after lung transplantation.
(A) The graft lungs and (B) contralateral lungs of the lung transplant groups (open square) and the sham-operated groups (open circle). Each point and bar represents the mean ± SD. *$P < 0.05$, compared with the baseline. #$P < 0.05$, differences between groups by repeated-measure analysis of variance.

Table Tumor necrosis factor (TNF)-α concentrations in bronchoalveolar lavage fluid after lung transplantation in a canine model

	Transplant group (n = 4)		Sham-operated group (n = 2)	
	Transplanted lung	Contralateral lung	Left lung	Right lung
TNF-α concentration (pg/mL)	0.24 ± 0.43	0.64 ± 0.43	3.18 ± 4.46	0.80 ± 1.12

Date are shown as mean ± SD

the lung transplant group (Fig. 6A). In addition, there was marked inflammatory infiltration and interstitial thickening in the contralateral lungs (Fig. 6B). By contrast, there were no significant findings in the bilateral lungs of the sham-operated group (Fig. 6C & D). Moreover, immunohistochemical staining showed significant TNF-α expression in the capillary endothelial cells of the grafted lungs after 18 hours of ischemia and 5 hours reperfusion, whereas there was no TNF-α expression in the sham-operated group (Fig. 7). TNF-α expression was also observed in alveolar macrophages in the grafted and contralateral lungs of the transplantation group, but not in the alveolar macrophages of the sham-operated group (Fig. 7, inset).

DISCUSSION

In a canine lung transplantation model, the present study demonstrated safe and successful serial ELF collection from transplanted lungs using BMS. Serial BMS revealed temporal changes in TNF-α concentration over a 5-hour period, corresponding to ischemia-reperfusion lung injury after lung transplantation. By contrast, the TNF-α concentration in BAL fluid was less than one-hundredth of that in ELF obtained using BMS. Therefore, serial ELF collection using BMS would be useful to elucidate the pathophysiology of posttransplant lung disorders and to understand the temporal changes in lung condition that occur after transplantation. To the best of our

knowledge, this is the first study to adopting BMS to investigate pulmonary disorder after lung transplantation.

As mentioned earlier, TBLB and BAL fluid collection can induce or exacerbate existing lung injuries, especially after transplant. Therefore, they are both considered unsuitable for serial ELF collection after lung transplantation. Furthermore, the significant dilution of BAL samples can significantly hamper the detection threshold and accuracy of biomarker measurements.

Severe ischemia-reperfusion injury was induced in a canine model of lung transplantation using 12 hours of cold ischemia and 6 hours of warm ischemia to validate BMS for lung transplantation. Histological findings showed marked infiltration of inflammatory cells in the grafted lungs compared with the sham-operated lungs. Moreover, blood gas analyses showed severe hypoxygenation and slight acidosis related to ischemia-reperfusion lung injury. Despite this condition, serial BMS could be performed to collect ELF from transplanted lungs without any adverse effects.

In the present study, serial BMS did not affect hemodynamics after the initiation of reperfusion, and the systolic arterial pressure and heart rate remained stable throughout the study period. The slight elevation in the mean PAP and peak airway pressure were most probably associated with the ischemia-reperfusion lung injury. Moreover, BMS probe insertion was minimally invasive and did not damage the bronchial anastomo-
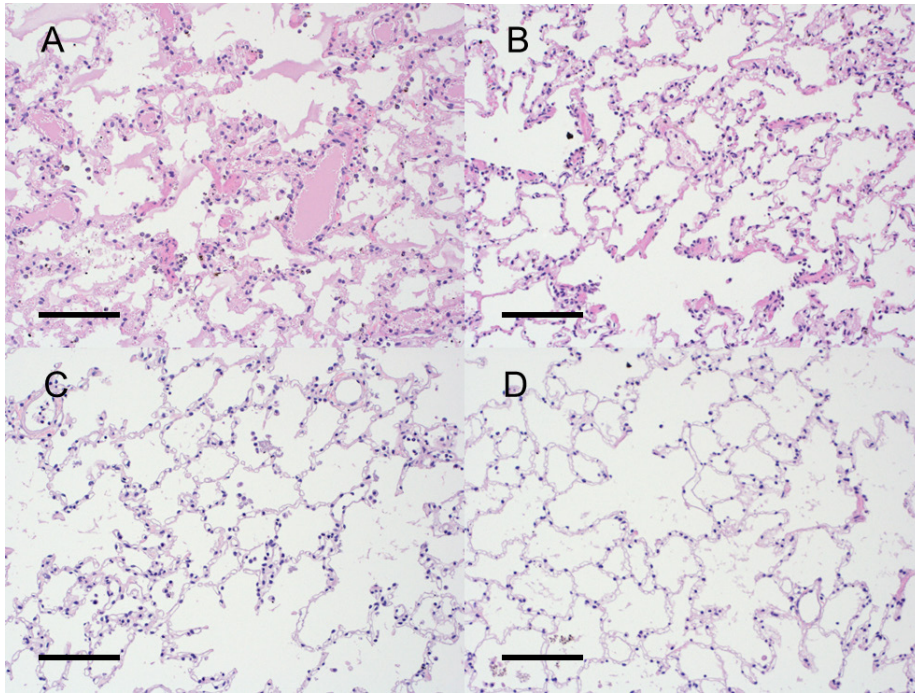
Fig. 6 Representative histological sections of canine lungs after lung transplantation and 6 hours of reperfusion.
(A) The graft lung. (B) The contralateral lung of the lung transplant group. (C) The left lung of the sham-operated group. (D) The contralateral lung of the sham operation group. Bars = 200 μm. Hematoxylin and eosin stain.
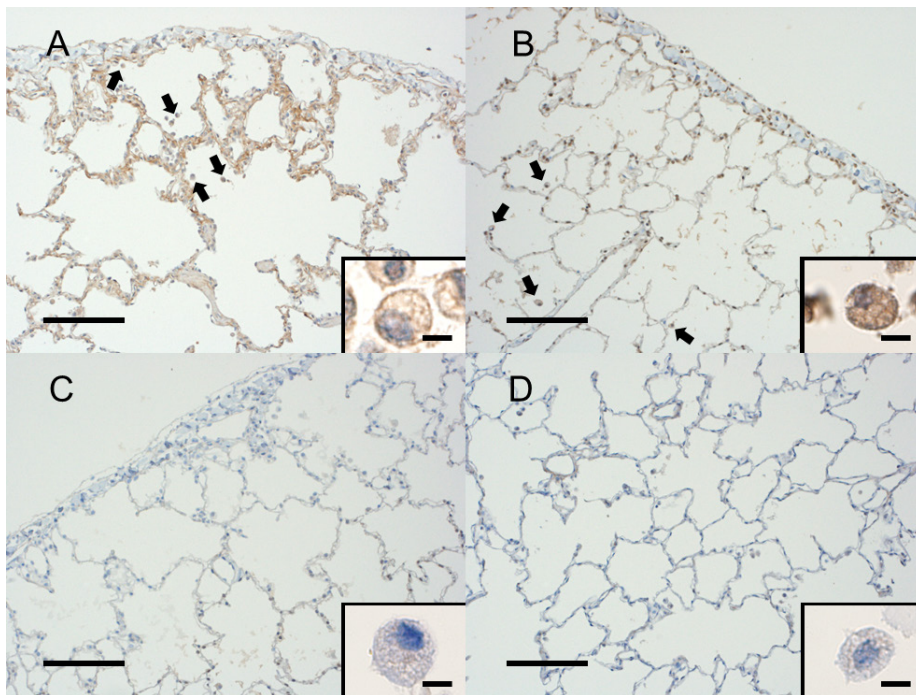

Fig. 7 Immunohistochemistry for tumor necrosis factor (TNF)-α on the transplanted canine lung after 18 hours of ischemia and 5 hours of reperfusion.
(A) The graft lung. (B) The contralateral lung of the lung transplant group. (C) The left lung of the sham-operated group. (D) The contralateral lung of the sham-operated group. Bars = 200 μm. Arrows indicate alveolar macrophages. Representative macrophages of each group are shown in the insets. Bars = 5 μm.

sis or cause hemorrhage, even when the probe was inserted into the segmental or subsegmental bronchi beyond the anastomosis of the left main bronchus. Furthermore, after the experiment, there was no slackness on the anastomosis of the pulmonary artery or left atrium.

In the present lung transplantation model, the volume of ELF collected using BMS ranged from 1–18 μL. This is because if the probe does not achieve adequate contact with the distal bronchial wall within 10 seconds of insertion, then the ELF adsorption into the polyurethane tip is minimal and the amount of ELF collected is insufficient. This error could be reduced by the use of three probes for each collection point and using averages values of the triplicate samples, as recommended. In the transplant group, the BMS ELF volumes collected in the graft and contralateral lungs tended to be greater than those collected in the sham-operated group, and there was a significant difference between the grafted and sham-operated lungs. This might reflect the hyperpermeability of the pulmonary vessels and edema caused by because of ischemia-reperfusion lung injury.

In the transplant group, temporal changes in ELF TNF-α concentrations, which have been associated with ischemia-reperfusion lung injury [15], were observed in both the grafted and contralateral lungs. By contrast, TNF-α levels in the sham-operated group were very low and barely detectable. It is reasonable that significant elevations in TNF-α were detected in specimens sampled from the injury foci because TNF-α would be produced by macrophages, endothelial cells, and fibroblasts in the grafted lungs [14, 15]. Furthermore, in the transplant group, elevated ELF TNF-α levels were sustained over a 5-hour period after the start of reperfusion in both the grafted and contralateral lungs. Temporal changes in systemic TNF-α, an early phase cytokine, vary according to the animal species and nature of the insult, but they typically reach a peak within 2 hours and decline within 4 hours of an initial insult [18, 19]. The present findings suggest that, after lung transplantation, the characteristics of the ischemia-reperfusion injury result in sustained elevations of TNF-α in the distal airways for an extended period.

All the ELF samples were collected from the caudal lobe bronchus in dogs positioned in the right decubitus position throughout the surgery and reperfusion procedures. Therefore, it is unlikely that there would be a significant characteristic difference in the ELF between the caudal and other lobes. However, in lung transplantation patients, there might be characteristic differences in the ELF between the lower and upper lobes because of the effects of gravity. This issue will be examined in a future clinical study.

In the comparison of BAL and BMS, it is believed that the BAL fluid reflects the condition of the alveolar areas rather than the bronchial areas, and in contrast, that ELF collected by BMS represents the bronchial areas rather than the alveolar areas. However, in the present study, immunohistochemistry showed that the most significant expressions of TNF-α were seen in the alveolar capillary endothelium and alveolar macrophages. Nevertheless, dynamic changes in TNF-α

levels were detected in the ELF collected from the distal bronchi of the grafted or contralateral lungs of the transplantation group. The TNF-α concentration in ELF was higher than 100 times greater than that in BAL fluid. Depending on the cytokine or molecule focused upon, BMS analysis of ELF might reflect not only the condition of the conducting portion of the airway but also that of the respiratory portion. Therefore, the characteristics of BMS and BAL should be considered and compared depending on the entity being assessed [20].

Ischemia-reperfusion injury in posttransplant graft dysfunction involves a complicated network of cytokines and chemokines [15, 21]. TNF-α is an upstream regulator of the network that causes organ dysfunction by affecting the expression of cell adhesion molecules and inflammatory mediators such as interleukin (IL)-1β, IL-6, IL-8, IL-10, and interferon (IFN)-γ. However, few studies have performed serial molecular biological measurements to determine the underlying pathophysiology of lung dysfunction after transplantation [22]. In future studies, we plan to apply BMS for serial ELF collection in the clinical setting of lung transplantation. This would enable discovery of the pathognomonic temporal changes in cytokine and chemokine expression. Furthermore, BMS could also be applied to investigate acute or chronic rejection that can also worsen the outcome of lung transplantation.

For the comparison of BMS and blood sampling, analysis of the ELF collected using BMS might reflect the lung condition more closely compared with serum analysis. Although serum measurements of biological molecules associated with lung injury are easy to perform, previous studies have indicated that the serum cytokine levels are low. For example, the serum TNF-α concentration in dogs with ischemia-reperfusion lung injury after lung transplantation were less than one-tenth of those in our ELF measurements [20]. While the cytokine kinetics associated with posttransplant lung injuries between the lungs and circulating blood are not clearly understood, judging by the concentration gradient, cytokines that are produced in the injured lungs might leak into the systemic circulation. Future studies comparing the serum and ELF might help to elucidate cytokine kinetics during posttransplant lung injury.

In conclusion, BMS was a safe and efficient technique for serial ELF collection in a canine model of lung transplantation. It permitted the collection of multiple samples over a significant duration without causing adverse effects. BMS was minimally invasive and could reduce the need to sacrifice animals used to study the mechanisms of pulmonary disorder after lung transplantation. BMS might also be useful for ELF collection in lung transplant patients, although further studies would be needed for validation in the clinical setting.

DISCLOSURES

The authors have no conflict of interest to disclose.

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