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Original Article

Ultrastructural alteration of pulmonary tissue under conditions of high oxygen concentration

Hirohisa Yano[1*], Ayumi Kuroda[1*], Hideshi Okada[1*], Hiroyuki Tomita[2], Kodai Suzuki[1], Chihiro Takada[1], Hirotsugu Fukuda[1], Yuki Kawasaki[1], Isamu Muraki[1], Yugo Wakayama[1], Soichiro Kano[1], Yuto Tamaoki[1], Ayane Nishio[1], Risa Inagawa[1], So Sampei[1], Ryo Kamidani[1], Yoshinori Kakino[1], Ryu Yasuda[1], Yuichiro Kitagawa[1], Tetsuya Fukuta[1], Takahito Miyake[1], Norihide Kanda[1], Nagisa Miyazaki[3], Tomoaki Doi[1], Takahiro Yoshida[1], Akio Suzuki[4], Shozo Yoshida[1], Shinji Ogura[1]

[1]Department of Emergency and Disaster Medicine, Gifu University Graduate School of Medicine, Gifu, Japan; [2]Department of Tumor Pathology, Gifu University Graduate School of Medicine, Gifu, Japan; [3]Department of Internal Medicine, Asahi University School of Dentistry, Mizuho, Gifu, Japan; [4]Department of Pharmacy, Gifu University Hospital, Gifu, Japan. [*]Equal contributors.

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Abstract: Objective: To determine the structure of pulmonary tissue under conditions of high oxygen concentration. Methods: Ten-week-old C57BL male mice and control mice were exposed to 100% oxygen and to room air for 72 hours, respectively. To follow the progression of lesions, the mice were sacrificed at 6, 12, 24, 48, and 72 hours after 100% oxygen administration. Lung specimens obtained from these mice underwent morphologic analysis and immunofluorescence studies. We used scanning and transmission electron microscopy to determine the ultrastructure of the pulmonary capillaries, including the endothelial glycocalyx. To visualize the endothelial glycocalyx, we performed lanthanum nitrate staining. Results: The survival rate of the 100% oxygen administration group was 5% (2/40) and that of the control group was 100%. Perivascular cavity enlargement was detected 12 hours after 100% oxygen administration and expanded over time. Ultrastructural analysis using electron microscopy revealed collapsed alveoli and pulmonary capillary wall and alveolar wall thickening in the 100% oxygen group. The pulmonary capillary endothelial glycocalyx was injured in the 100% oxygen group. The perivascular cavity decreased in mice that were returned to room air after 48 hours of 100% oxygen administration. Conclusion: High-concentration oxygen causes perivascular cavity enlargement; this is thought to be a special characteristic of high oxygen damage. In addition, high-concentration oxygen may be involved in pulmonary endothelial glycocalyx injury.

Keywords: Hyperoxic acute lung injury, microcirculation disorder, endothelial glycocalyx

Introduction

In critically ill patients with circulatory shock and severe pulmonary infection, oxygen supply is one of the most effective medical interventions. Although oxygen plays a key role in the synthesis of adenosine triphosphate, its chemical characteristics lead to strong oxidizing properties, resulting in damage to many biological molecules [1]. Oxygen toxicity results from the detrimental effects of breathing molecular oxygen at increased partial pressures. In severe cases, there is cell damage and death in several organ systems. Hyperoxic acute lung injury refers to a variety of physiologic effects, including impaired pulmonary gas exchange, decreased vascular perfusion, inflammation, and attenuated ATP synthesis mediated by low mitochondrial oxygen consumption [2].

In the vascular system, hyperoxia increases systemic vascular resistance [3-5], and hyperoxia-related vasoconstriction impairs tissue oxygen delivery in patients with sepsis [6]. Endothelial cells play important roles including gas exchange and nutrition supply. Their surfaces are covered by the endothelial glycocalyx, which maintains microcirculatory homeostasis by modulating vascular tone and leukocyte adhesion.

Pulmonary oxygen toxicity presents as severe pulmonary inflammation, ultimately leading to hemorrhagic pulmonary edema, associated

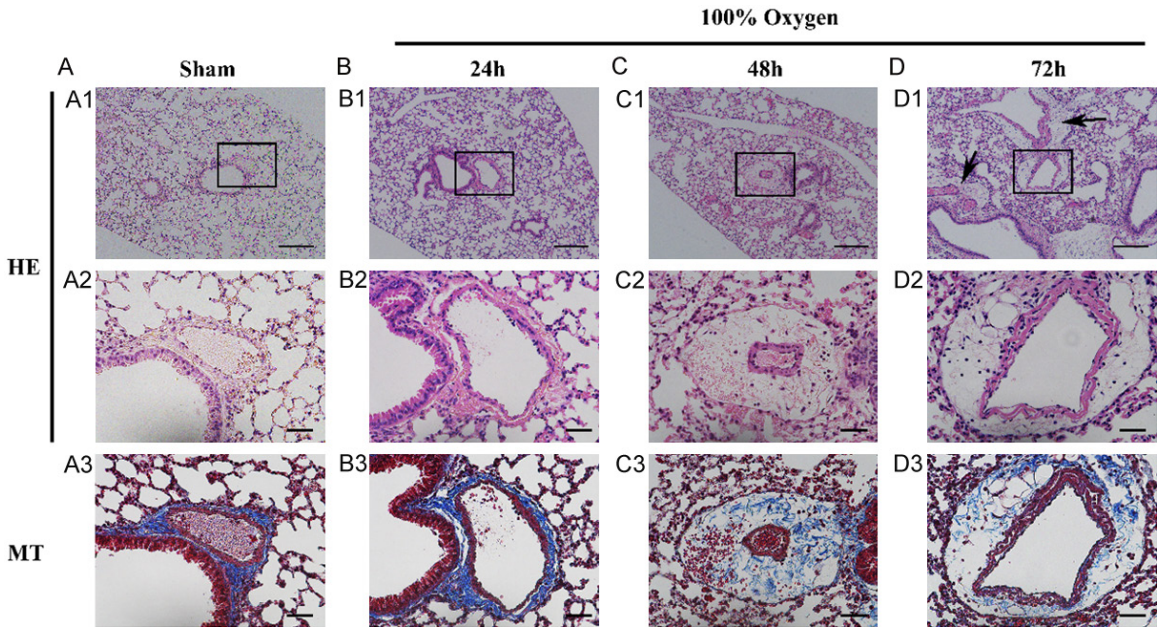

Figure 1. Histological analysis of hyperoxic lung. (A) Lung specimen under normoxic conditions. (A1 and A2) show hematoxylin and eosin-stained lung specimens. (A2) is an expanded view of the area in the white square in (A1). (A3) is a Masson's trichrome-stained lung specimen. (B-D) Lung specimens under 100% oxygen incubation for 24, 48, and 72 hours, respectively. (B1, B2, C1, C2, D1 and D2) are hematoxylin and eosin-stained lung specimens. (B2, C2 and D2) are expanded views of the area in the white squares in (B1, C1 and D1), respectively. (B3, C3 and D3) are Masson's trichrome-stained lung specimens. Perivascular cavity enlargement was detected 24 hours after 100% oxygen administration and increased as time passed. Bars = 100 μm in (A1, B1, C1 and D1). Bars = 20 μm in (A2, A3, B2, B3, C2, C3, D2 and D3).

with production of excess reactive oxygen species [7, 8]. The endothelial glycocalyx may be injured in hyperoxic acute lung injury. Reactive oxygen species damage lung tissue, including the alveolar epithelium and pulmonary vascular endothelial cells, directly or indirectly through inflammatory cells such as macrophages.

The aim of the present study was to document the ultrastructural morphology of hyperoxic acute lung injury including endothelial glycocalyx degradation.

Materials and methods

In vivo studies

This study conformed to the Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Research Committee of Gifu University (Gifu, Japan). Ten-week-old male C57BL6 mice were obtained from Chubu Kagaku Shizai Co. Ltd. (Nagoya, Japan).

High concentration oxygen incubation

In the experimental 100% oxygen incubated model, the hyperbaric chamber for animals was filled with 100% oxygen. The chamber was vented to maintain 100% oxygen at atmospheric pressure. The mice were maintained under 100% oxygen conditions for 72 hours. Control mice were maintained in room air for 72 hours under similar conditions. The survival rates were determined every 12 hours, until 72 hours after 100% oxygen incubation, followed by sacrifice of the surviving mice and collection of lung specimens.

Next, to determine if there could be a reversal effect caused by return to normal air, 48 hours after incubation in 100% oxygen, some mice were maintained under room air conditions for 48 hours. The survival rate was determined every 12 hours, up to 96 hours.

Histopathologic examination

Lungs were harvested from mice 24, 48, and 72 hours after 100% oxygen administration

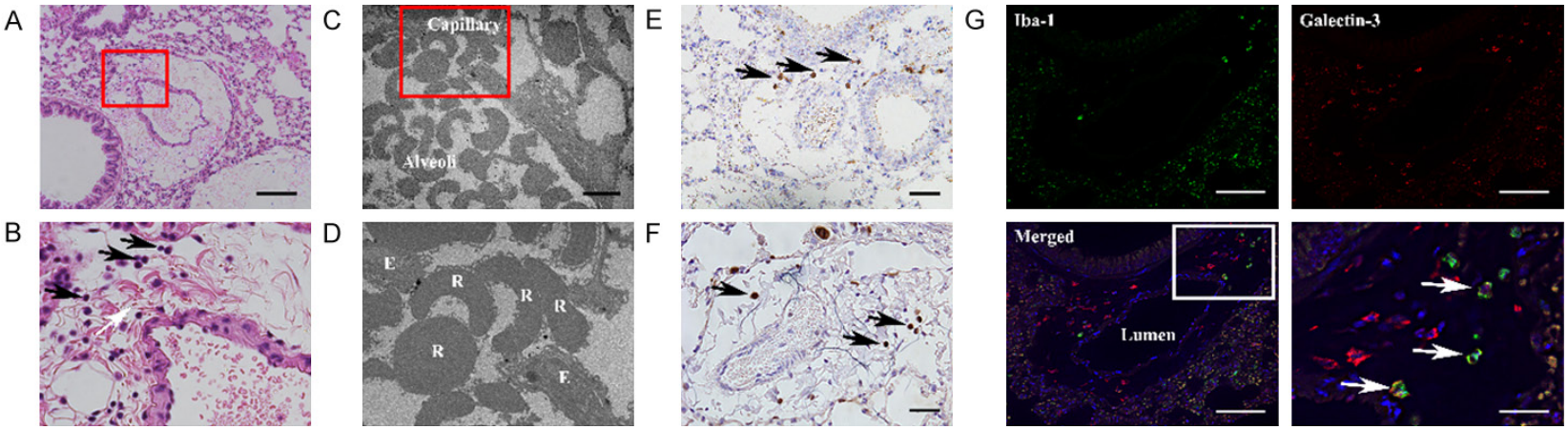

Figure 2. Cell components in the perivascular cavity. (A) Lung specimen 48 hours after 100% oxygen incubation. Bar = 100 μm. (B) An expanded view of the area in the red square in (A). Black arrows indicate inflammatory cells and the white arrow indicates red blood cells in the perivascular cavity. (C) TEM imaging of the lung 48 hours after 100% oxygen incubation. Bar = 5 μm. (D) An expanded view of the area in the red square in (C). Red blood cells flow out from the pulmonary capillaries to the alveoli between the pulmonary capillary endothelial cells. R: red blood cells. E: pulmonary capillary endothelial cells. (E) Immunohistochemical analysis of Iba-1 in the lung 48 hours after 100% oxygen incubation. Arrows indicate Iba-1 positive cells. Bar = 50 μm. (F) Immunohistochemical analysis of galectin-3 in the lungs 48 hours after 100% oxygen incubation. Arrows indicate galectin-3 positive cells. Bar: 50 μm. (G) Double immunofluorescence analysis of Iba-1 and galectin-3 in the lung 48 hours after 100% oxygen incubation. The magnified image is the expanded view of the white-boxed area in the merged image. White arrows indicate both of Iba-1 and galectin-3 positive cells. Bars = 20 μm.

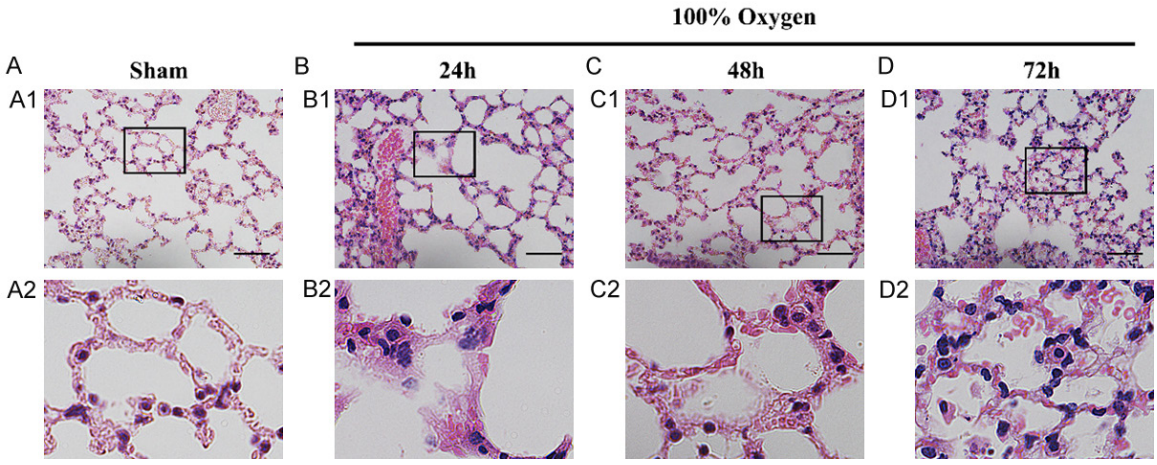

Figure 3. Histology of pulmonary capillaries under hyperoxic conditions. (A) Hematoxylin and eosin-stained lung specimen under normoxic conditions. (A2) is an expanded view of the area in the white square in (A1). (B-D) Hematoxylin and eosin-stained lung specimens under 100% oxygen incubation for 24, 48, and 72 hours, respectively. (B2, C2 and D2) is an expanded view of the area in the black squares in (B1, C1 and D1), respectively. The alveolar wall becomes edematous 24 hours after 100% oxygen administration and ameliorates as the time passed. Red blood cells flow into the alveoli from 48 hours after 100% oxygen incubation. Bars = 100 μm.

and were fixed with phosphate buffered saline (PBS) containing 10% formalin. Paraffin sections were then de-paraffinized and rehydrated. Finally, slides were counterstained with hematoxylin and eosin. To determine the areas of fibrosis, Masson's trichrome staining was also performed.

Quantitative assessment of the extravascular area

Quantitative assessment of the extravascular area of the capillary lumens was performed on six randomly selected capillary vessels in hematoxylin and eosin-stained images using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). In each vessel, the areas of the extravascular cavity and intravascular lumen area were measured. Next, the ratio of the extravascular to intravascular lumen area was calculated by dividing the extravascular area by the intravascular area.

In vivo assay for blood vessel permeability

Ten-week-old male mice were first intraperitoneally administered a sterile solution of Evans blue in PBS (WAKO, Japan, 100 μg/kg) as described previously [9]. The mice were maintained in the 100% oxygen chamber for 48 hours (n = 6), 24 hours after Evans blue injection. Control mice were maintained in normal air for 48 hours in a similar fashion. In vivo assays for blood vessel permeability were performed as described previously [9].

Immunohistochemistry for Iba-1 and galectin-3

After de-paraffinization, 4-μm thick sections were made and incubated with primary antibodies against Iba-1 (019-19741; Wako Pure Chemical, Osaka, Japan), which is an indicator of macrophage activation [10], and/or galectin-3/Mac2 (galectin-3) (14-5301; eBioscience Co., Ltd., San Diego, USA), which is a β-galactoside-binding lectin that is important in cell proliferation and regulation of apoptosis [11-13]. The target proteins were visualized using the VECTASTAIN Elite ABC system (Vector Laboratories) or secondary antibodies (Alexa Fluor 488 and 568, Invitrogen) and Hoechst nuclear stain.

Electron microscopy

Electron microscopic analysis of the endothelial glycocalyx was performed as described previously [14, 15].

Data analysis

Data are expressed as mean ± standard error of measurement. The Student's two-tailed *t*-test was used for comparing the two groups, and survival data were analyzed using the log-rank test; $P < 0.05$ was considered significant.

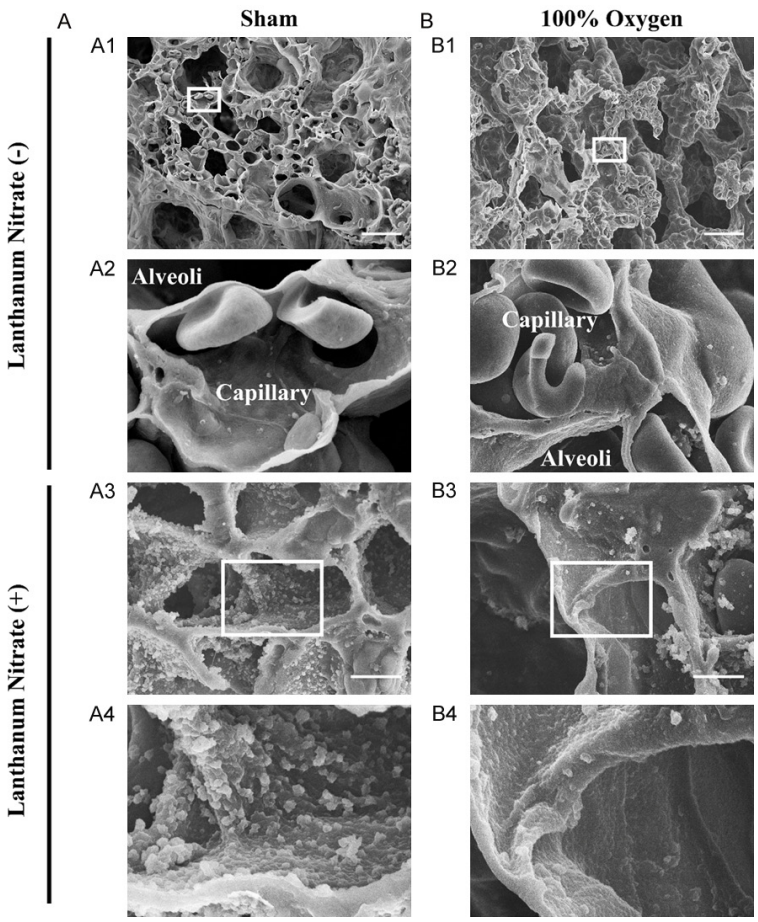

Figure 4. Pulmonary capillary alteration on scanning electron microscopic imaging. (A) Lung specimens under normoxic conditions. (A1 and A2) are images without lanthanum nitrate. Bar = 20 μm. (A2) is an expanded image of the area in the white square in (A1). The walls of alveoli between the alveoli and capillaries are thin. (A3 and A4) are images of the endothelial glycocalyx using lanthanum nitrate. Bar = 2 μm. (A4) is an expanded image of the area in the white square in (A3). The endothelial glycocalyx covers the surface of pulmonary capillary endothelial cells. (B) Lung specimens 48 hours after 100% oxygen incubation. (B1 and B2) are images without lanthanum nitrate. Bar = 20 μm. (B2) is an expanded image of the area in the white square in (B1). The wall of alveoli between the alveoli and capillaries is thick compared with that in the normoxic lung. (B3 and B4) are images of the endothelial glycocalyx using lanthanum nitrate. Bar = 2 μm. (B4) is an expanded image of the area in the white square in (B3). The endothelial glycocalyx degrades and flows away from the surface of the pulmonary capillary endothelial cells, and the surface of the endothelial cells is exposed to the vascular lumen.

All calculations were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

Results

High-concentration oxygen causes lung injury

The survival rate of mice 72 hours after 100% oxygen incubation was 5% (2/40), and that of the control group was 100% (Supplementary Figure 1). By histologic analysis, in the control group, there was fibrosis around the vessels, particularly the smooth muscle layer of the arteries under the normal air condition (**Figure 1A**). Perivascular cavity enlargement was detected 24 hours after 100% oxygen administration (**Figure 1B**), and this increased over time (**Figure 1C**, **1D**). The ratio of the extravascular-to-intravascular lumen area increased with time (Supplementary Figure 2). At 72 hours after 100% oxygen incubation, the perivascular cavity was expanded and connected with nearby blood vessels. This phenomenon is thought to be a special characteristic of high oxygen damage. In this cavity, there were components such as red blood cells, fibroblasts, and inflammatory cells (**Figure 2A** and **2B**). TEM revealed red blood cells extravasated from capillaries to alveoli, probably because of endothelial cell injury (**Figure 2C** and **2D**). These inflammatory cells were Iba-1-positive, indicating that these were macrophages residing in alveoli (**Figure 2E**). There were also galectin-3-positive cells (**Figure 2F**), substantially associated with fibrosis [16]. The cells were identified as expressing both Iba-1 and galectin-3 (**Figure 2G**).

Pulmonary edema was detected 24 hours after 100% of oxygen exposure compared with room air exposure (**Figure 3A** and **3B**). Red blood cells leaked into the alveolar space 48 hours and 72 hours after 100% oxygen incubation (**Figure 3C** and **3D**), suggesting that exposure to 100% oxygen injured the pulmonary capillaries.

Pulmonary microvascular injury under hyperoxic conditions

To quantitatively analyze blood vessel permeability, we measured extravasation of Evans blue. In pulmonary capillaries, the amount of

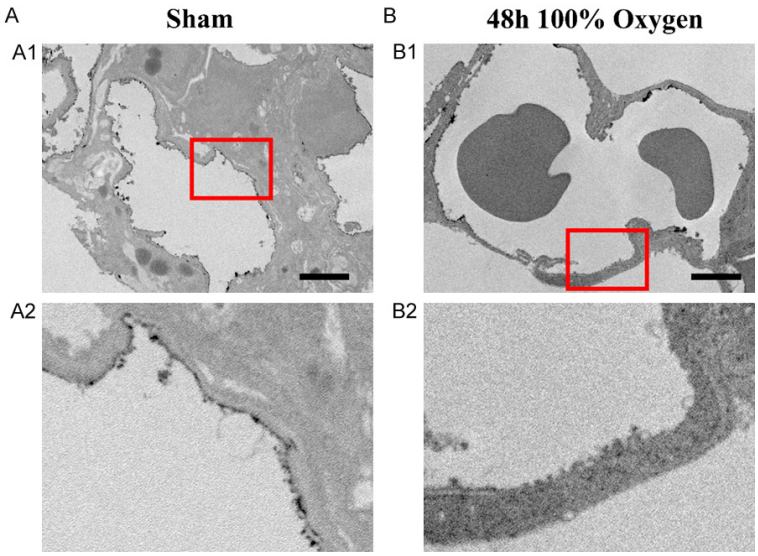

Figure 5. Pulmonary capillary endothelial glycocalyx alteration on transmission electron microscopic imaging. A pulmonary capillary (A) under normoxic conditions and (B) 48 hours after 100% oxygen incubation. (A2) and (B2) show expanded views of the area in the red squares in (A1 and B1), respectively. Although the endothelial glycocalyx covers the surface of the pulmonary capillary endothelial cells under normoxia, 100% oxygen incubation caused endothelial glycocalyx injury. Bars = 2 μm.

Evans blue extravasation was significantly higher in the hyperoxic group (6.5 ± 0.8 μg/mL/g) than in the control group (3.7 ± 0.3 μg/mL/g, $P < 0.01$; Supplementary Figure 3).

To address microvascular injury, ultrastructural analysis was performed. Ultrastructural analysis using scanning electron microscopy revealed collapsed alveoli and pulmonary capillary wall and alveolar wall thickening in mice in the 100% oxygen administration group (**Figure 4A1**, **4A2**, **4B1** and **4B2**). Endothelial glycocalyx covered the entire surface of the luminal side of the endothelium in control mice (**Figure 4A3** and **4A4**). In contrast, in hyperoxic mice, the endothelial glycocalyx was degraded, and the surface of the endothelium was exposed to the lumen of the capillaries 48 hours after 100% oxygen incubation (**Figure 4B3** and **4B4**).

On TEM, the endothelial glycocalyx appeared injured, and wall thickness was greater 48 hours after 100% oxygen exposure than after equal exposure to normal air (**Figure 5**). Red blood cells were seen leaking from vessels to the alveolar space through gaps between the endothelial cells.

Recovery of enlargement of the perivascular cavity

Next, we determined whether enlargement of the perivascular cavities was reversible by exposure to room air 48 hours after 100% oxygen incubation. We found that the perivascular cavity area decreased in mice subjected to room air for 24 hours after having been exposed to 100% oxygen for 48 hours (**Figure 6**).

Discussion

We found that hyperoxia was associated with microcirculatory injury due to endothelial glycocalyx degradation. The endothelial glycocalyx is composed of glycosylated proteins and coats all healthy vascular endothelium. It plays a pivotal role in microvascular physiology by maintaining microvascular tone and endothelial permeability and by regulating adhesion/migration of leukocytes [17-19]. In particular, the endothelial glycocalyx is strongly associated with vascular permeability [20, 21]. The endothelial glycocalyx is disrupted not only by acute stressors such as sepsis, but also by chronic conditions such as diabetes and hypertension. Its disruption exposes endothelial cells to blood, and vascular permeability is increased [15, 22]. Our current findings suggest that hyperoxia also causes endothelial glycocalyx injury. Because the endothelial glycocalyx contributes to the microvascular tone, its degradation may be associated with vasoconstriction.

As described above, microcirculation may be hindered by endothelial glycocalyx injury, subsequently causing extravasation of fluid. Since hyperoxia also injures pulmonary tissue, interstitial fluid flow may be straggled. Therefore, it is presumed that the extravascular cavity may be expanded.

We also found that hyperoxic acute lung injury is reversible after re-incubation in normal air, consistent with findings of previous reports [7, 23]. It was previously reported that the endo-

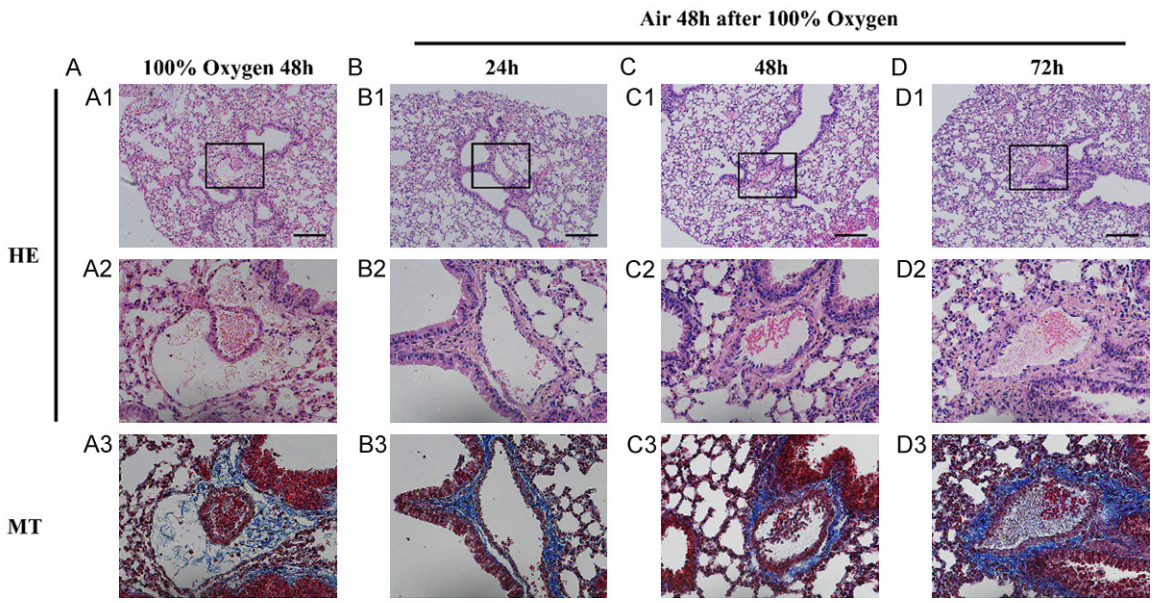

Figure 6. Recovery of enlargement of the perivascular cavity. (A) Lung specimen 48 hours after 100% oxygen incubation. (A1 and A2) show hematoxylin-eosin stained lung specimens. (A2) is an expanded view of the area in the white square in (A1). (A3) is a Masson's trichrome-stained lung specimen. (B-D) Lung specimens under normoxia 24, 48, and 72 hours after 100% oxygen incubation, respectively. (B1, B2, C1, C2, D1 and D2) are hematoxylin and eosin-stained lung specimens. (B2, C2 and D2) show expanded views of the areas in the white squares in (B1, C1 and D1), respectively. (B3, C3 and D3) are Masson's trichrome-stained lung specimens. The perivascular cavity area has already decreased in mice subjected to room air for 24 hours, following 48 hours of 100% oxygen administration. Bars = 100 μm.

thelial glycocalyx layer returned to normal after resolution of injury by lipopolysaccharides in a short period of time [14]. Presumably, returning to normoxia from the hyperoxic state may cause endothelial glycocalyx recovery, subsequently restoring the microcirculation of the lung. This result also suggests that hyperoxia injures the endothelial glycocalyx.

We identified Iba-1-positive cells in the perivascular cavity 48 hours after 100% oxygen incubation. Iba-1 is expressed in activated macrophages that are found in inflammatory tissues. It was previously reported that Iba-1 is an indicator of macrophage activation [10]. These cells also expressed galectin-3, which is a β-galactoside-binding lectin important in cell proliferation and regulation of apoptosis [11-13]. Galectin-3 is expressed by immune cells such as macrophages and plays an important role in diverse physiologic functions. Galectin-3 is expressed on the cell surface and is secreted by injured and inflammatory cells. It was recently reported that galectin-3 was a useful biomarker for cardiac disorders such as cardiac inflammation and fibrosis, depending on the specific pathogenesis [16]. In the present study, we found that Iba-1- and galectin-3-positive cells migrated into the perivascular cavity 48 hours after 100% oxygen incubation. We speculate that activated macrophages migrate to treat the perivascular cavity that results from the microcirculatory disorder.

In clinical practice, oxygen therapy is often administered to critically ill patients, in whom endothelial injury may already exist when the oxygen therapy was started. In addition, if artificial ventilation is required, the alveoli would be damaged by positive pressure ventilation. Therefore, in these cases, we speculate that microcirculatory injury occurs earlier. Conversely, if it is possible to protect the endothelial glycocalyx, hyperoxic acute lung injury might attenuate under hyper oxygen therapy.

We should note as a study limitation that as the present study is descriptive, further research is required to clarify the implicated mechanisms.

Perspectives

While oxygen therapy is one of the most effective medical interventions, hyper oxygen supply

causes microcirculation injury via endothelial glycocalyx degradation. Conversely, endothelial glycocalyx protection might attenuate lung injury from hyper oxygen supply.

Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hideshi Okada, Department of Emergency and Disaster Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan. Tel: +81-58-230-6448; Fax: +81-58-230-6451; E-mail: hideshi@gifu-u.ac.jp

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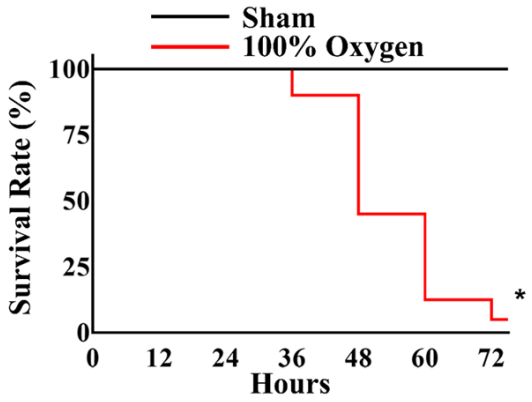
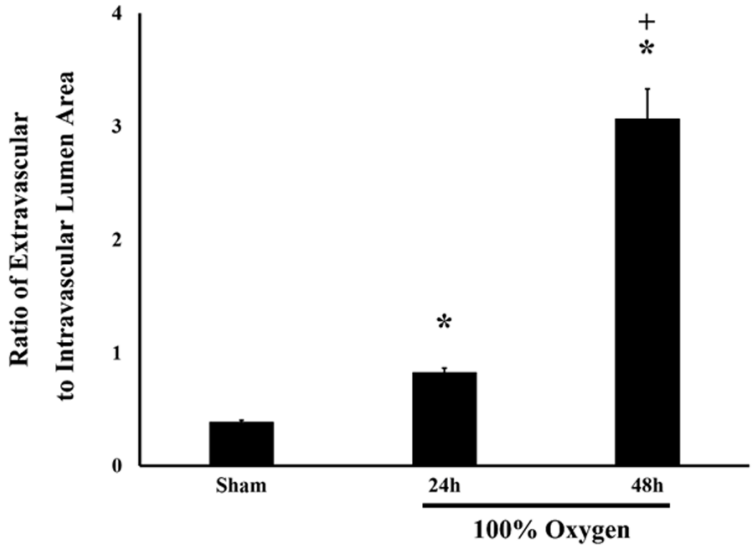
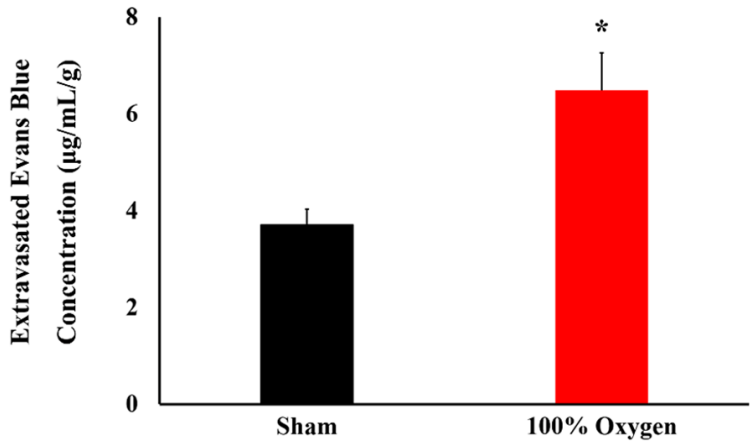


Supplementary Figure 1. Survival rate. Kaplan-Meier survival curves for the mice under normoxia (n = 10) and 100% oxygen incubation (n = 40). *P < 0.05 vs. mice under normoxia.



Supplementary Figure 2. Ratio of the extravascular to intravascular lumen area. *P < 0.05 vs. mice under normoxia. +P < 0.05 vs. mice 48 hours after 100% oxygen incubation.



Supplementary Figure 3. Extravascular Evans blue concentration. *P < 0.05 vs. mice under normoxia.