Original Article

Gene expression profiles in respiratory settings in rats under extracorporeal membrane oxygenation

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Background: Venovenous extracorporeal membrane oxygenation (VV-ECMO) is an effective lung protection strategy that avoids ventilator-induced lung injury. However, appropriate respiratory settings for VV-ECMO are yet to be established. This study aimed to elucidate the effects of ventilation under VV-ECMO using a newly developed rat VV-ECMO model and analyzed gene expression profiles.

Methods: Rats were assigned to three groups of five rats each: spontaneous breathing, conventional-protective ventilation, and ultra-protective ventilation. The conventional protective and ultraprotective ventilation groups received volume-controlled ventilation at a frequency of 60 and 20 beats/min, with tidal volumes of 6 and 3 mL/kg, respectively. VV-ECMO was performed at a pump flow rate of 20–30 mL/kg/min. At 120 min post initiation of VV-ECMO, rats were euthanized, and their lungs were harvested. Changes in gene expression were assessed using microarray analysis.

Results: Gene expression profile analyses revealed lowest expression of inflammation/immune promotion, cytotoxicity, and cell proliferation related genes (*Defa5*, *Prg2*, *Siglec8*, *Atf3*, *Rnd1*, *Ctsg*, and *Gc*), and the highest expression of inflammation/immune suppression related genes (*Pp2d1*) in the spontaneous breathing group as compared to that in the other two mechanical ventilation groups.

Conclusions: The findings of this study demonstrated that spontaneous breathing was the least invasive respiratory setting under VV-ECMO. Further, mechanical ventilation may be associated with lung injury even at low ventilation frequency and tidal volume.

Keywords: Gene expression; extracorporeal membrane oxygenation (ECMO); lung; ventilation; rat

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Introduction

Acute respiratory distress syndrome (ARDS) occurs in approximately 10% of Intensive Care Unit admissions, of which 80% require invasive ventilator management. The mortality rate in severe cases can be as high as 46% (1). Consequently, lung protective ventilation to prevent ventilator-induced lung injury is an important parameter of invasive ventilator management for ARDS (2-5). Cases of severe ARDS that respond inadequately to conventional ventilator management are increasingly being treated with venovenous extracorporeal membrane oxygenation (VV-ECMO) (6,7). Further, the efficacy of VV-ECMO in critically ill patients was evident in coronavirus disease 2019-associated ARDS during the global pandemic (8). Extracorporeal support with VV-ECMO therefore is an efficient lung protection strategy that avoids lung injuries consequent to ventilator-induced hyperoxia and hyperventilation pressure (9-12).

While VV-ECMO permits reduction of the intensity of mechanical ventilation to avoid ventilator induced lung injury (VILI), the appropriate respiratory settings are yet to be established (13). Ultra-protective ventilation that combines very low tidal volumes and low respiratory rates has been attempted to enable further reduction of VILI (14).

Highlight box

Key findings

- Gene expression profile analyses revealed lower expression of inflammation/immune promotion, cytotoxicity, and cell proliferation related genes, and the higher expression of inflammation/immune suppression related genes in the spontaneous breathing group as compared to that in the other mechanical ventilation groups.

What is known and what is new?

- Venovenous extracorporeal membrane oxygenation (VV-ECMO) is an effective lung protection strategy that avoids ventilator-induced lung injury.
- However, appropriate respiratory settings for VV-ECMO are yet to be established. In this study, we investigated the effects of ventilation under VV-ECMO using a newly developed rat VV-ECMO model and analyzed gene expression profiles.

What is the implication, and what should change now?

- The results of this study support the benefit weaning off ventilator support, and maintenance of spontaneous breathing during VV-ECMO in clinical practice, from a molecular and biological perspective.

A subsequent validation study, however, reported no observable decrease in biotrauma in VV-ECMO-supported ARDS patients, along with a trend towards higher 60-day mortality, post 48 hours of ultra-protective ventilation, despite the significant reduction in mechanical power (15). In contrast, ultra-protective ventilation was found to reduce histological lung injury and matrix metalloproteinase activity, as well as prevent myofibroblast marker expression in an ECMO-supported pig model of ARDS (16). Additionally, self-inflicted lung injury is a major concern during spontaneous respiration in patients with ARDS. Injurious transpulmonary pressure swings may occur due to high inspiratory drive and large tidal volumes during spontaneous respiration in these patients. Further, increased transmural pulmonary vascular pressure swings due to the inspiratory effort may increase vascular permeability (17). Basic animal experiments on gene expression are therefore essential to elucidate the mechanisms underlying these conflicting results.

The present study investigated the effects of a ventilator on VV-ECMO on a newly developed rat VV-ECMO model and analyzed gene expression profiles (18). We present this article in accordance with the ARRIVE reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1661/rc).

Methods

Ethical considerations

A protocol was prepared before the study without registration. This study was approved by the Institutional Animal Care and Use Committee of Jikei University (approval number 2018-070). The animal experiments were carried out to the Guidelines for the Proper Conduct of Animal Experiments of the Science Council of Japan.

Animals

Male Sprague-Dawley rats with a mean weight of 518 g and a range of 389–622 g were used for this study (CLEA Japan Inc., Tokyo, Japan). Male rats were only used to address concerns regarding variability in gene expression. Rats were housed under controlled conditions of 12-12 h light-dark cycle, room temperature of 22±2 ℃, and humidity of 55%±10%, with free access to food (CE-2, CLEA Japan Inc.) and water. Rats were allocated to three groups in turn: spontaneous breathing, conventional protective ventilation,

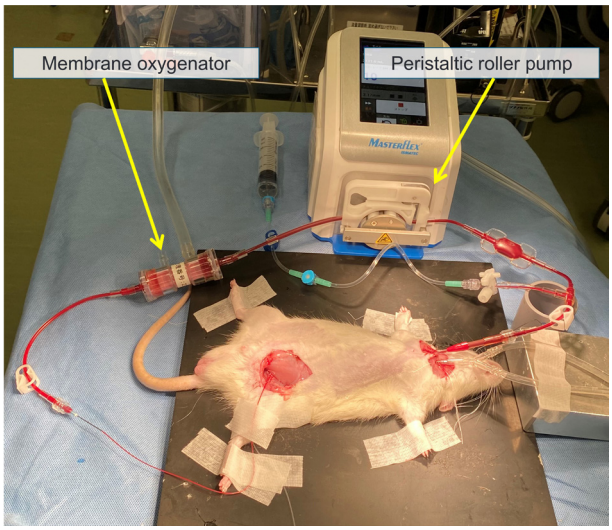

Figure 1 The experimental model of venovenous extracorporeal membrane oxygenation of rat. The model comprised a 17-G catheter for venous uptake cannulated via the right external jugular vein into the right atrium, a polyvinyl chloride tube line, a peristaltic roller pump, a membrane oxygenator, and an SP-31 polyethylene tube for venous return cannulation of the left femoral vein.

and ultraprotective ventilation. The experimental unit was a single animal. There was no prior sample size calculation, and the allocation continued until the cumulative total number of cases that were able to reach an endpoint of maintaining VV-ECMO for 120 min reached to five in each group. Cases in which the preparation of the VV-ECMO failed or in which the VV-ECMO could not be maintained for 120 min due to problems with the tubes or due to bleeding were excluded.

Intervention

Anesthesia

A mixture of medetomidine-midazolam-butorphanol was intraperitoneally injected as an anesthetic at a dose of 0.15 mg/kg of medetomidine (Kyoritsu Seiyaku, Tokyo, Japan), 2.0 mg/kg of midazolam (Sandoz K.K. Ltd., Tokyo, Japan) and 2.5 mg/kg of butorphanol (Meiji Seika, Tokyo, Japan). A quarter of the initial dose was subsequently administered every hour to maintain anesthesia. During anesthesia, the rats were kept warm with a heating mat.

Ventilation

Rats were placed in supine position. The conventional-protective ventilation and ultra-protective ventilation groups were subjected to a cut down the mid-neck and were intubated via tracheostomy using a 16-G catheter (Terumo Corp., Tokyo, Japan). Mechanical ventilation was initiated using a Respirator Model SN-480-7 (Shinano Seisakusho, Tokyo, Japan). While conventional protective ventilation was volume-controlled at a frequency of 60/min, with a tidal volume of 6 mL/kg body weight, ultra-protective ventilation was volume-controlled at a frequency of 20/min, with a tidal volume of 3 mL/kg body weight. The spontaneous breathing group underwent the mid-neck cut, without subsequent tracheostomy or intubation.

Preparation of VV-ECMO

The left inguinal region was excised, followed by exposure of the left femoral vein, which was cannulated using an SP-31 polyethylene tube (Natsume Seisakusho Co., Ltd., Tokyo, Japan) that was meant to serve as the venous return cannula for the VV-ECMO system. Heparin sodium was then administered at a dose of 500 IU/kg via the cannula. Next, the right neck region was cut to expose the right external jugular vein. A 17-G catheter (Supercath Clampcath; Togo-Medkit Co., Ltd., Tokyo, Japan) was then cannulated through the right external jugular vein into the right atrium as a conduit for venous uptake. The VV-ECMO system included a polyvinyl chloride tube line (Y-F line, Senko Medical Instrument Mfg. Co., Ltd., Japan, Tokyo), a membrane oxygenator with a membrane area of 0.03 m^2 (Senko Medical Instrument Mfg. Co., Ltd.), and a peristaltic roller pump (Masterflex Ismatec, Avantor, Radnor, PA, USA). The Tube line and membrane oxygenator were primed with 7.5 mL of physiological saline containing 10 IU/mL heparin. *Figure 1* illustrates the experimental conditions.

Experimental groups

The experimental groups are depicted in *Figure 2*. VV-ECMO was maintained with 20–30 mL/kg/min blood flow and 1 L/min sweep gas flow of 100% oxygen. The cannula in the external jugular vein was repositioned and saline solution was administered as necessary in cases of poor venous uptake. Post 120 min of VV-ECMO, 4 mL blood samples were collected, and the rats were subsequently euthanized by cervical dislocation.

Tissue processing

The left lung was harvested from euthanized rats and divided in to two parts. The superior part was frozen immediately as

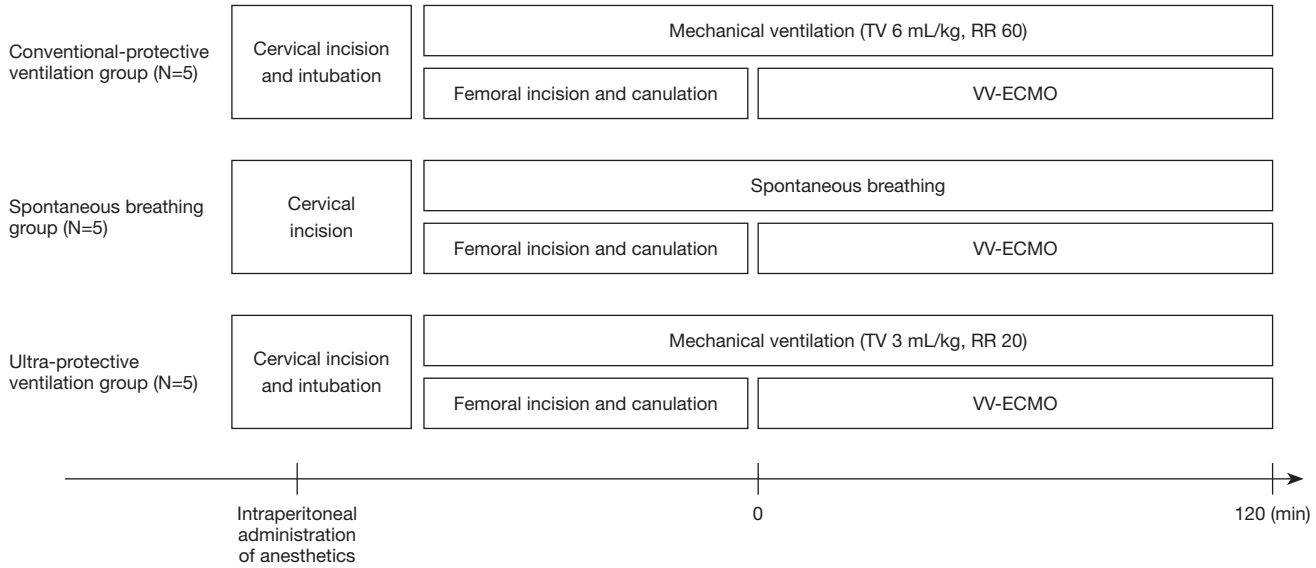

Figure 2 Timelines for each group. Rats were randomly assigned to three groups of five rats each: spontaneous breathing, conventional protective ventilation, and ultraprotective ventilation. Post intraperitoneal administration of anesthetics, a cervical incision was made in all groups, and tracheostomy and intubation were performed in the conventional protective ventilation and ultraprotective ventilation groups. While conventional protective ventilation was volume-controlled at an RR of 60/min, with a TV of 6 mL/kg body weight, ultra-protective ventilation was volume-controlled at an RR of 20 /min, with a TV of 3 mL/kg body weight. Post making a femoral incision, VV-ECMO was initiated with a pump flow rate of 20–30 mL/kg/min. Rats were euthanized after 120 min, followed by harvesting of the left lung. TV, tidal volume; RR, respiratory rate; VV-ECMO, venovenous extracorporeal membrane oxygenation.

such in liquid nitrogen and stored at −80 ℃ till further use. The inferior part was placed in 4% paraformaldehyde, prior to dehydration using 99.5% ethanol solution, followed by embedding in paraffin, and sectioning into 4 μm slices. The sections were then stained with hematoxylin and eosin. Blood was centrifuged at 1,500 rpm for 10 min to separate and collect the serum, which was stored at −80 ℃.

Analyses

Histological evaluation

Neutrophils were counted in 10 randomly selected fields at ×400 high power field. All slides were evaluated by two independent investigators who were blinded to the data. The average neutrophil count in each sample was recorded.

Serum cytokine assays

Serum inflammatory cytokines were evaluated using a magnetic multiplex assay (Rat Magnetic Luminex® Assay, Rat Premixed Multi-Analyte Kit, R&D Systems, Minneapolis, MN, USA). The Luminex assay was performed using a Luminex 200 instrument (Luminex Corporation, Austin, TX, USA), according to the manufacturer's protocol.

Gene expression profile analysis

Total RNA was extracted from rat lungs using an miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The concentration and purity of extracted RNA were assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA), respectively. Samples with an A260/A280 ratio >1.8 and an RNA integrity number (RIN) >7.0 were considered pure and suitable for further analysis. Microarray analysis was performed using a 3D-Gene Rat Oligo chip 20k (Toray Industries Inc., Tokyo, Japan). Total RNA was labeled with Cy5 using the Amino Allyl MessageAMP II aRNA Amplification Kit (Applied Biosystems, Carlsbad, CA, USA). Cy5-labeled aRNA pools were hybridized for 16 h in the hybridization buffer in accordance with the manufacturer's instructions. Hybridization signals were obtained using a 3D-Gene Scanner 3000 (Toray Industries Inc.) and processed using the 3D-Gene Extraction software (Toray Industries Inc.). Signals detected for each gene were

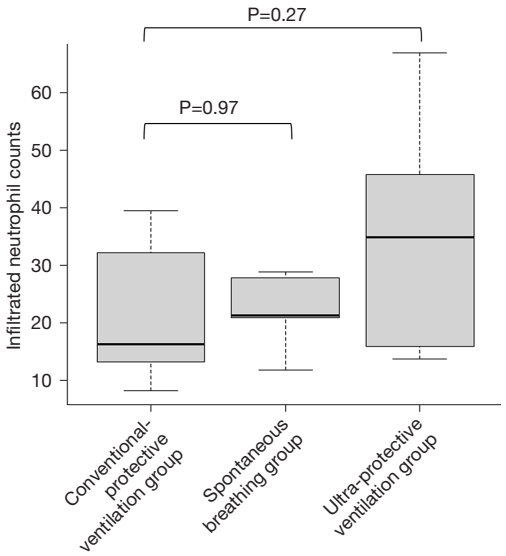

Figure 3 A comparison of intrapulmonary infiltrated neutrophil counts in each group. The mean and standard deviation of neutrophil counts in conventional-protective ventilation, spontaneous breathing, and ultra-protective ventilation groups were 21.9±13.3, 22.1±6.8, and 35.4±22.2, respectively. There was no significant difference in the counts of the spontaneous breathing and ultraprotective ventilation groups and those of the conventional protective ventilation group (P=0.97 and P=0.27, respectively).

normalized using the global normalization method.

Statistical analyses

Data are presented as mean ± standard deviation (SD). Depending on the data, either the Fisher's exact test, Chi-squared test, or *t*-test was used to assess correlations between variables and groups. If the test value was above or below the upper or lower test limits, the analysis was performed using the upper or lower test limits. All analyses were performed using JMP (version 13.0.0; SAS Institute, Cary, NC, USA), and a P value <0.05 was considered significant. At the time of data analysis, each case was not blinded.

Results

Experimental group

A total of 30 rats were used, of which eight died due to VV-ECMO preparation failure and seven died due to problems with the tubes or bleeding during VV-ECMO. Finally, five cases in each group reached the endpoint of maintaining VV-ECMO for 120 min. The experiments were conducted between December 2020 and April 2021 with an average frequency of 6 cases per month at The Jikei University School of Medicine.

Histological evaluation

Figure 3 demonstrates the comparison of intrapulmonary infiltrated neutrophil counts in each group. The mean and SD of neutrophil counts in the conventional-protective ventilation, spontaneous breathing, and ultra-protective ventilation groups were 21.9±13.3, 22.1±6.8, and 35.4±22.2, respectively. There was no significant difference between the neutrophil counts of the spontaneous breathing and ultraprotective ventilation groups in comparison to that of the conventional protective ventilation group (P=0.97 and P=0.27, respectively).

Serum cytokine assays

Table 1 shows the comparison of serum inflammatory cytokines in the spontaneous breathing and ultra-protective ventilation groups with that of the conventional protective ventilation group. Although the L-selection was lower in the conventional protective ventilation group than that in the other groups, there were no significant differences evident in the other cytokines between the groups.

Gene expression profile analyses

Tables 2-4 depict the comparisons of lung mRNA expression between each group. An increase of ≥4.0-fold change (FC) or decrease of ≤0.25 FC in mRNA expression has been listed.

Discussion

Our study elucidated gene expression profiles in rat lungs under VV-ECMO with and without ventilator usage.

The spontaneous breathing group had the lowest expression levels of genes related to inflammation/immune promotion, cytotoxicity, and cell proliferation, and the highest expression levels of genes related to inflammation/immune suppression in the three groups studied. These findings suggest that spontaneous breathing may be the least invasive ventilation setting under VV-ECMO.

Expression levels of *Defa5*, an inflammatory peptide contained within neutrophil granules (19), *Prg2*, contained within eosinophil granules and involved in the immune

Table 1 Serum inflammatory cytokines in spontaneous breathing group and ultra-protective ventilation group comparing to conventional-protective ventilation group

Serum inflammatory cytokines	Conventional-protective ventilation group (n=5)	Spontaneous breathing group (n=5)		Ultra-protective ventilation group (n=5)	
		Value	P value	Value	P value
CXCL2	24±18	20±8	0.63	16±0	0.35
CXCL3	16±6	13±0	0.35	13±0	0.35
GM-CSF	7±2	8±3	0.41	6±0	0.29
ICAM-1	31,034±10,726	21,805±6,139	0.13	20,480±3,951	0.07
IFN-gamma	530±303	739±385	0.37	510±208	0.91
IL-1 alpha	45±39	36±8	0.62	32±14	0.49
IL-1 beta	13±15	8±4	0.48	6±1	0.28
IL-2	20±0	20±0	>0.99	20±0	>0.99
IL-4	4±0	4±0	>0.99	4±0	>0.99
IL-6	272±414	68±9	0.30	70±12	0.31
IL-10	52±67	109±133	0.41	29±21	0.49
IL-13	3±1	3±0	0.43	3±0	0.35
IL-18	177±283	102±72	0.58	109±138	0.64
L-selectin	59,092±9,849	69,315±0	0.049	69,315±0	0.049
TIMP-1	5,831±2,374	5,154±1,199	0.59	5,670±2,162	0.91
TNF-alpha	43±7	49±20	0.58	40±0	0.35
VEGF	19±5	22±8	0.54	21±5	0.69

Values are listed with mean ± standard deviation (pg/mL). GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Table 2 Expressed lung mRNAs in the spontaneous breathing group comparing to the conventional-protective ventilation group

mRNAs symbol	Description	FC
Increased expression (FC ≥4.0)		
Pp2d1	Protein phosphatase 2C-like domain containing 1	4.17
Decreased expression (FC ≤0.25)		
Defa5	Defensin alpha 5	0.11
Hba-a1	Hemoglobin alpha, adult chain 1	0.14
Prg2	Proteoglycan 2	0.15
Fam111a	Family with sequence similarity 111, member A	0.18
Siglec8	Sialic acid binding Ig-like lectin 8	0.23
Atf3	Activating transcription factor 3	0.24
Rnd1	Rho family GTPase 1	0.25
Ctsg	Cathepsin G	0.25
Gc	Group specific component	0.25

FC, fold change.

Table 3 Expressed lung mRNAs in the spontaneous breathing group comparing to the ultra-protective ventilation group

mRNAs symbol	Description	FC
Increased expression (FC ≥4.0)		
Lyzl6	Lysozyme-like 6	8.33
Jade2	Jade family PHD finger 2	5.88
Pp2d1	Protein phosphatase 2C-like domain containing 1	5.56
Golgb1	Golgin B1	5.00
Ypel1	Yippee-like 1	4.55
Zc3h13	Zinc finger CCCH type containing 13	4.17
Sumo2	Small ubiquitin-like modifier 2	4.17
Decreased expression (FC ≤0.25)		
Olr46	Olfactory receptor 46	0.23
Defa5	Defensin alpha 5	0.23
Klf9	Kruppel-like factor 9	0.24

FC, fold change.

Table 4 Expressed lung mRNAs in the ultra-protective ventilation group comparing to the conventional-protective ventilation group

mRNAs symbol	Description	FC
Increased expression (FC ≥4.0)		
Olr46	Olfactory receptor 46	4.53
Klf9	Kruppel-like factor 9	4.03
Decreased expression (FC ≤0.25)		
Mycn	V-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog	0.15
Hist1h2bh	Histone cluster 1, H2bh	0.20
LOC102551819	U3 small nucleolar RNA-associated protein 14 homolog B-like	0.20
Jade2	Jade family PHD finger 2	0.21
Pxmp4	Peroxisomal membrane protein 4	0.22
Ccl2	Chemokine (C-C motif) ligand 2	0.22
Gc	Group specific component	0.22
Golgb1	Golgin B1	0.23
LOC259246	Alpha-2u globulin PGCL1	0.24
Enc1	Ectodermal-neural cortex 1	0.24
Edn3	Endothelin 3	0.25

FC, fold change.

response (20), *Siglec8*, which promotes eosinophil apoptosis (21), *Atf3*, which is produced by stress stimuli and regulates metabolism and immune responses (22), *Rnd1*, which increases pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) levels (23), *Ctsg*, a component of neutrophil granules that promotes

immune cell migration, increases vascular permeability, induces thrombus formation and acts on connective tissue remodeling (24), and *Gc*, which has a macrophage-activating effect (25), were decreased in the spontaneous breathing group as compared to that in the conventional-protective ventilation group. In contrast, expression of *Pp2d1*, which regulates various cellular events, including, proliferation, differentiation, and stress responses, was increased in the former as compared to that in the latter (26). These findings imply that spontaneous breathing is more lung protective than conventional-protective ventilation.

A comparison of gene expression profiles between the spontaneous breathing and ultraprotective ventilation groups revealed a decrease in expression levels of *Defa5* and *Klf9*, which increases with oxidative stress and promotes lung fibrosis in the former as compared to that in the latter (27). In contrast, expression levels of *Pp2d1*, *Zc3h13*, which is reported to be involved in mRNA methylation and is a protective factor against interstitial lung inflammation (28), *Sumo2*, which regulates excessive immune responses (29), and *Jade2*, which is involved in chromatin remodelling, cell cycle progression, cell regeneration, and DNA damage response (30). were increased in the spontaneous breathing group in comparison to that in the ultra-protective ventilation group, suggesting that the former was better protected against lung damage that the latter.

Comparisons between the ultra-protective ventilation and conventional-protective ventilation groups revealed upregulation of certain genes associated with inflammation and immunity with concomitant downregulation of others. Expression of *Gc*, *Ccl2*, which recruits monocytes, memory T cells, and dendritic cells to sites of inflammation post tissue injury or infection (31), and *Enc1*, which reduces the oxidative stress response and acts in cytoprotection (32), and *Jade2* were decreased in the ultra-protective ventilation group as compared to that in the conventional-protective ventilation group. In contrast, *Klf9* expression was increased in the former as compared to that in the latter. Each ventilator setting was found to result in different gene expression profiles. Based on the results alone, although if any one was a more non-invasive setting remained unclear, mechanical ventilation itself may cause lung tissue injury, even if the ventilation frequency and tidal volume are low.

These results support weaning off ventilator support, and maintenance of spontaneous breathing during VV-ECMO in clinical practice, from a molecular and biological perspective.

Our study has several limitations. First, although the rat model attempted to replicate the VV-ECMO environment in human patients as closely as possible, the present study was limited by the unresolved pathophysiological response differences between actual clinical patients and the model. The physiological values set in the experiments are therefore not guaranteed to be appropriate for human clinical practice. Second, assessment of changes due to exposure over long periods (days) the actual period of adaptation to VV-ECMO was not possible. We were thus able to monitor changes due to exposure only over short periods (120 min), which may not have resulted in discernable differences in serum cytokine levels or histopathological findings between the groups. Third, this study did not use positive end-expiratory pressure (PEEP) and did not evaluate the ideal setting of PEEP during VV-ECMO. The setting of PEEP in patients with severe ARDS is controversial. Recently, the use of some degree of high PEEP has been recommended in patients with severe ARDS (33). High PEEP may be associated with a low ratio of overdistended regions to recruited regions, indicating more homogeneous ventilation and improvement in ventilation-perfusion matching (34). On the other hand, a study showed that high PEEP increased maximal hyperinflation and tidal hyperinflation, decreased lung compliance and did not decrease tidal recruitment/de-recruitment (35). In clinical practice, a concrete ideal value suitable for all patients cannot be determined, and considering the findings of this study that mechanical ventilation, even with lower tidal volume and lower respiratory rate, may induce a more inflammatory response than spontaneous breathing, it will be necessary to dynamically adjust the ventilatory settings in each patient according to various parameters, not only oxygenation, when using mechanical ventilation under VV-ECMO. Fourth, Arterial oxygen partial pressure was not compared between groups in this study. Hypoxia-inducible transcription factor-1 and NF-kappaB, which are induced under hypoxic conditions, induce the expression of inflammatory cytokines and chemokines were reported (36,37). Latent differences in arterial oxygen partial pressure between the groups might influence the differential expression of genes involved in inflammatory and immune responses. Fifth, potential confounders were not controlled for because randomization was not performed.

Conclusions

The gene expression analysis results in this animal study revealed that spontaneous breathing is the least invasive respiratory setting under VV-ECMO, on account of its

association with the lowest expression of inflammation/immune-promotion and cytotoxicity related genes, and the highest expression of inflammation and immune-suppression related genes. Additionally, mechanical ventilation may be associated with lung injury even if the ventilation frequency and tidal volume are low.

Acknowledgments

None.

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1661/rc

Data Sharing Statement: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-1661/dss

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was reviewed and approved by the Institutional Animal Care and Use Committee of Jikei University (approval number 2018-070). The animal experiments were carried out to the Guidelines for the Proper Conduct of Animal Experiments of the Science Council of Japan.

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