

Effects of Hypercapnia on Acute Cellular Rejection after Lung Transplantation in Rats

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ABSTRACT

Background: Hypercapnia alleviates pulmonary ischemia–reperfusion injury, regulates T lymphocytes, and inhibits immune reaction. This study aimed to evaluate the effect of hypercapnia on acute cellular rejection in a rat lung transplantation model.

Methods: Recipient rats in sham-operated (Wistar), isograft (Wistar to Wistar), and allograft (Sprague–Dawley to Wistar) groups were ventilated with 50% oxygen, whereas rats in the hypercapnia (Sprague–Dawley to Wistar) group were administered 50% oxygen and 8% carbon dioxide for 90 min during reperfusion ($n = 8$). Recipients were euthanized 7 days after transplantation.

Results: The hypercapnia group showed a higher oxygenation index (413 ± 78 vs. 223 ± 24), lower wet weight-to-dry weight ratio (4.23 ± 0.54 vs. 7.04 ± 0.80), lower rejection scores (2 ± 1 vs. 4 ± 1), and lower apoptosis index (31 ± 6 vs. 57 ± 4) as compared with the allograft group. The hypercapnia group showed lower CD8 (17 ± 4 vs. 31 ± 3) and CD68 (24 ± 3 vs. 43 ± 2), lower CD8⁺ T cells (12 ± 2 vs. 35 ± 6), and higher CD4/CD8 ratio (2.2 ± 0.6 vs. 1.1 ± 0.4) compared to the allograft group. Tumor necrosis factor- α (208 ± 40 vs. 292 ± 49), interleukin-2 (30.6 ± 6.7 vs. 52.7 ± 8.3), and interferon- γ (28.1 ± 4.9 vs. 62.7 ± 10.1) levels in the hypercapnia group were lower than those in allograft group. CD4, CD4⁺ T cells, and interleukin-10 levels were similar between groups.

Conclusions: Hypercapnia ameliorated acute cellular rejection in a rat lung transplantation model. (ANESTHESIOLOGY 2018; 128:130–9)

ACUTE cellular rejection is a type of organ dysfunction initiated by cell-mediated immunity in lung-transplant recipients.¹ During acute cellular rejection, T cells are involved in the modulation of antigen-specific responses, whereas macrophages mediate the actual effector function; CD68⁺ macrophages, which constitute the most abundant subtype, play a particularly important role.^{2,3} Imbalance between pro-inflammatory (interleukin-1 β , tumor necrosis factor- α , and interferon- γ) and anti-inflammatory (interleukin-4 and interleukin-10) cytokines modulates the immune response to acute cellular rejection.⁴ The former cytokines are related to rejection, whereas the latter are involved in immune tolerance.⁵

Ischemia–reperfusion injury is considered a part of the innate immune response mediated through Toll-like receptor-mediated signaling pathways, which induces a persistent phase of antigen processing and presentation.⁶ A subsequent severe immune response causes acute cellular rejection by inducing abnormal expression of major histocompatibility complex and by up-regulating local cytokines. Inhibition of lung ischemia–reperfusion injury may alleviate acute cellular rejection.

Therapeutic hypercapnia refers to the use of inhalational carbon dioxide or reduction of ventilation to achieve a desired level of hypercapnic acidosis.⁷ Hypercapnia may alleviate lung injury caused by ischemia–reperfusion,⁸ endotoxins,⁹ mechanical ventilation,¹⁰ adult respiratory distress syndrome (ARDS),¹¹ and chronic pulmonary hypertension.¹²

What We Already Know about This Topic

- “Permissive hypercapnia” accompanying low tidal volume ventilation is accepted clinical practice. However, “therapeutic hypercapnia,” in which carbon dioxide is primarily elevated, is a laboratory intervention with mixed impact.

What This Article Tells Us That Is New

- Randomized exposure to 8% inspired carbon dioxide resulted in preserved lung function, less lung injury, and lower indices of cellular rejection 7 days after lung allograft (different strains) transplantation in a rat model.

Our previous studies showed that hypercapnia may inhibit ischemia–reperfusion injury in the brain¹³ and liver¹⁴ and reduce the inflammatory response after one-lung ventilation in lobectomy patients.⁷

Hypercapnia inhibits cell-mediated immune responses by decreasing leukocyte subpopulation counts and by reducing release of proinflammatory cytokines.¹⁵ A carbon dioxide–induced acidic microenvironment regulates a variety of cell functions, including production of adenosine triphosphate, cell proliferation, and apoptosis.^{16,17} Our previous research confirmed that hypercapnia mediates the immunoregulation of T cells by indirectly inhibiting chemotactic factors and adhesion molecules and by directly suppressing CD28 and CD2 during lung ischemia–reperfusion injury.¹⁸ Therefore, we hypothesized that hypercapnia has a therapeutic effect

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against acute cellular rejection and thus investigated related mechanisms in a rat model of lung transplantation.

Materials and Methods

Experimental Animals

Pathogen-free male Sprague–Dawley and Wistar rats weighing 200 to 250 g were purchased from Harbin Medical University (Harbin, Heilongjiang, China). All animal experiments were approved by the Institutional Animal Care and Use Committee at the Harbin Medical University. All procedures conformed to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Washington, D.C., 7th edition, 1996).

Experimental Setting

The study included four groups: sham-operated (Wistar), isograft (Wistar to Wistar), allograft (Sprague–Dawley to Wistar), and hypercapnia (Sprague–Dawley to Wistar). In total, 16 Sprague–Dawley rats were randomly assigned to two blocks of eight animals each, and 40 Wistar rats were assigned to five blocks of eight animals each, using a random number list generated by SPSS (version 18.0; SPSS, Inc., USA). Each block of Sprague–Dawley rats was randomly assigned to allograft and hypercapnia groups as donors. Among Wistar rats, one block was randomly assigned to the sham-operated group, two blocks were assigned to the isograft group as both donors and recipients, and the remaining two blocks were assigned to the allograft and hypercapnia groups as recipients.

Orthotopic left-lung transplantation was performed using the cuff technique as previously described.¹⁹ Donor rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg; Shuogang Biotechnological Company, China), intubated through a tracheostomy and ventilated with 50% oxygen (balance nitrogen) and a tidal volume (V_T) of 8 to 10 ml/kg at a rate of 40 to 60 breaths/min. Arterial blood gas (ABG) analysis was allowed to equilibrate over a 10-min period. Subsequently, heparin (1,000 U/kg; Nanjing Biopharmaceutical Company, China) was injected intravenously, and a median thoracotomy was performed. The donor left lung was flushed with 20 ml of low- K^+ polybutadienyl lithium solution (prepared by Harbin Medical University) at 4°C at a perfusion pressure of 20 cm H_2O . The left lung was clipped and stored at 4°C in the perfusion solution for 2 h.

The recipient rats were anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneal injection) and vecuronium bromide (0.1 mg/kg, intravenous; Xianju Biopharmaceutical Company, China). These animals were intubated with a 14-gauge catheter and ventilated using the following settings: V_T = 8 to 10 ml/kg, rate = 40 to 60 breaths/min, fraction of inspired oxygen = 50%, and positive end-expiratory pressure (PEEP) = 2 cm H_2O . After a left thoracotomy, the left pulmonary arteries, veins, and bronchi were conjugated between donors and recipients, respectively. After initiation

of reperfusion, isograft and allograft groups received 50% O_2 (balance nitrogen) titrated to maintain arterial carbon dioxide tension ($Paco_2$) at 35 to 45 mmHg. Hypercapnia group received 50% O_2 and 8% CO_2 (balance nitrogen) to maintain $Paco_2$ within the range of 80 to 100 mmHg. This distinct ventilation regime for different groups was administered for 90 min. Oxygen and carbon dioxide concentration was monitored with a T8 Mindray monitor (Mindray Biotechnology, China). Rats in the sham-operated group were administered 50% O_2 (balance nitrogen) for 90 min and underwent a dummy surgical operation, which included thoracotomy, separation of hila, and closure of thorax.

The thorax was then closed with a chest drainage tube *in situ* after 90 min of reperfusion. Postoperative analgesia was achieved by incision closure using 2 ml of 0.5% ropivacaine (AstraZeneca AB, Sweden). All groups were ventilated with 50% O_2 (balance nitrogen) until restoration of spontaneous respiration. After recovery from anesthesia, the tracheal catheter and the chest drainage tube were removed. The rats were housed in the Animal Care Center in pathogen-free air conditions. Free access to clear rodent chow and water was allowed. No immunosuppressive therapy was administered during the duration of the experiment.

On postoperative day 7, chest x-rays of recipient rats were obtained under sedation with isoflurane (Abbott, United Kingdom). Then the animals were anesthetized with sodium pentobarbital (30 mg/kg) and ventilated with 50% O_2 (balance nitrogen; V_T = 8 to 10 ml/kg at a rate of 40 to 60 breaths/min). After 10 min of stable status, a thoracotomy and ABG analysis were performed. Flow cytometry and enzyme-linked immunosorbent assay were then performed using a femoral artery blood sample. The recipients were euthanized by exsanguination. The ratio of wet weight-to-dry weight of the upper lobe of the graft was measured after desiccation at 80°C for 1 week. The middle section was assessed with hematoxylin and eosin staining and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling staining. Immunohistochemical staining for CD4, CD8, and CD68 was performed on a section of the inferior lobe of the lung. Additionally, the right lung, liver, heart, and brain tissues were collected for hematoxylin and eosin staining. All study parameters were evaluated by experts in their respective fields who were blinded to the group identity.

Radiographic Evaluation

The x-rays were graded according to the aeration scores described elsewhere (range = 0 [opaque] to 6 [normal appearance]).²⁰

Apoptosis Assay

Lung parenchymal cell apoptosis was assessed by TdT-mediated dUTP nick end-labeling staining assay using the *in situ* cell death detection kit (Roche Diagnostics, Germany) according to the manufacturer's instructions. Cells with brownish yellow nuclear staining were considered positive.

Apoptosis index was calculated as the number of positive cells per 100 cells.

Hematoxylin and Eosin Staining and Rejection Scores

The tissues were fixed in paraformaldehyde and embedded in paraffin; 5- μ m-thick sections were prepared and stained with hematoxylin and eosin. Graft rejection was scored according to the clinical International Working Formulation.²¹

Immunohistochemistry

Immunohistochemical staining for CD4, CD8, and CD68 was performed according to the manufacturer's instructions (Abcam, United Kingdom) and evaluated by the percentage of positive staining. Cells with brown granules on the surface were defined as positive cells.

Flow Cytometry

Blood samples were double-stained with fluorescein phycoerythrin-CD4 or phycoerythrin-CD8 in combination with isothiocyanate-CD3 monoclonal antibodies (Invitrogen, USA) and analyzed by flow cytometry.

Enzyme-linked Immunosorbent Assay

Serum concentrations of tumor necrosis factor- α , interleukin-2, interleukin-10, and interferon- γ were detected by a commercial kit, as specified by the manufacturer (R&D Systems, USA).

Statistical Analysis

Sample size was based on our previously reported studies related to attenuation of lung graft injury^{22,23} and a study by Hirayama *et al.*²¹ on protection of acute lung graft rejection, which employed similar outcome measures. There were no missing or lost data or data that were excluded from the analysis.

The data are presented as means \pm SD. Statistical analyses were performed with SPSS (version 18.0). Multigroup comparisons were performed with one-way ANOVA followed by the Student–Newman–Keuls test. Results of ABG analysis (including the P_{aO_2}/F_{IO_2} , P_{aCO_2} , and pH levels) were assessed by two-way repeated measures ANOVA with time (three levels: baseline, at 90 min, and at 7 days after reperfusion) as a within-subject factor and group (four levels: sham-operated, isograft, allograft, and hypercapnia) as a between-subject factor. For multigroup comparisons for the values (P_{aO_2}/F_{IO_2} , P_{aCO_2} , and pH) at different time points (baseline, at 90 min, and at 7 days after reperfusion), we used the Student–Newman–Keuls *post hoc* test. Furthermore, in each group, we used the Student–Newman–Keuls *post hoc* test to perform repeated comparisons for the values of P_{aO_2}/F_{IO_2} , P_{aCO_2} , and pH at the time points of 90 min (or 7 days) after reperfusion against the baseline. Overall, significant differences with respect to time, group, and the interaction between time and group were determined by a two-tailed *P* value less than 0.05.

Results

There were no significant between-group differences with respect to P_{aO_2}/F_{IO_2} , P_{aCO_2} , and pH at baseline (fig. 1, A–C). The P_{aO_2}/F_{IO_2} in the hypercapnia group was significantly higher than that in the allograft group during reperfusion at 90 min (449 ± 59 *vs.* 302 ± 47) and on day 7 (413 ± 78 *vs.* 223 ± 24 ; both $P < 0.0001$). After 90 min of reperfusion, the P_{aCO_2} was obviously higher (92 ± 6 *vs.* 41 ± 5), whereas pH was markedly lower (7.11 ± 0.07 *vs.* 7.37 ± 0.05) in the hypercapnia group as compared to that in the allograft group (both $P < 0.0001$). However, this pattern was not observed on day 7 after transplantation. The wet weight-to-dry weight ratio in the hypercapnia group was lower than that in the allograft group (4.23 ± 0.54 *vs.* 7.04 ± 0.80 , $P = 0.002$; table 1).

Lung grafts from the sham-operated, isograft, and hypercapnia groups demonstrated good ventilation similar to that in the contralateral native (right) lung. In contrast, the graft in the allograft group exhibited impaired ventilation (fig. 1D). Aeration score in the hypercapnia group was similar to that in the isograft group (3 ± 1 *vs.* 4 ± 1 , $P = 0.705$; fig. 1E).

With respect to cell apoptosis, the number of apoptosis-positive cells in the pulmonary parenchyma was markedly reduced in the sham-operated, isograft, and hypercapnia groups. However, a greater number of positive cells were observed in the allograft group (fig. 2A). The sham-operated (12 ± 3 *vs.* 57 ± 4 , $P < 0.0001$), hypercapnia (31 ± 6 *vs.* 57 ± 4 , $P = 0.008$), and isograft (27 ± 5 *vs.* 57 ± 4 , $P = 0.001$) groups exhibited a markedly lower apoptosis index compared to the allograft group (fig. 2B).

In the grafts, CD4 staining was rarely distributed in all of the examined specimens from the four groups (fig. 3A). The sham-operated and isograft groups exhibited slight CD8 and CD68 staining. However, CD8 and CD68 staining were more frequently expressed in the allograft group but weakly expressed in the hypercapnia group (fig. 3, B and C). There were no significant differences with respect to CD4 staining between the four groups (fig. 3A). The CD8 and CD68 staining in the sham-operated (8 ± 2 *vs.* 31 ± 3 and 11 ± 1 *vs.* 43 ± 2 , both $P < 0.0001$), hypercapnia (17 ± 4 *vs.* 31 ± 3 , $P = 0.018$, and 24 ± 3 *vs.* 43 ± 2 , $P = 0.012$), and isograft (12 ± 3 *vs.* 31 ± 3 and 20 ± 2 *vs.* 43 ± 2 , both $P < 0.0001$) groups were lower than that in the allograft group (fig. 3, B and C).

In peripheral blood samples, no significant differences were observed with respect to the CD4⁺ T cell percentage in the sham-operated (28 ± 5 *vs.* 31 ± 6 , $P = 0.237$), isograft (30 ± 5 *vs.* 31 ± 6 , $P = 0.644$), and hypercapnia (30 ± 5 *vs.* 31 ± 6 , $P = 0.611$) groups as compared to that in the allograft group (fig. 4A). The percentage of CD8⁺ T cells was elevated in the allograft group but significantly decreased in the sham-operated (35 ± 6 *vs.* 11 ± 3), isograft (35 ± 6 *vs.* 15 ± 4), and hypercapnia (35 ± 6 *vs.* 12 ± 2) groups ($P < 0.0001$ for all; fig. 4B). Furthermore, the ratio of CD4⁺/CD8⁺ T cells in the hypercapnia group was higher than that in the allograft group (2.2 ± 0.6 *vs.* 1.1 ± 0.4 , $P = 0.020$; fig. 4C).

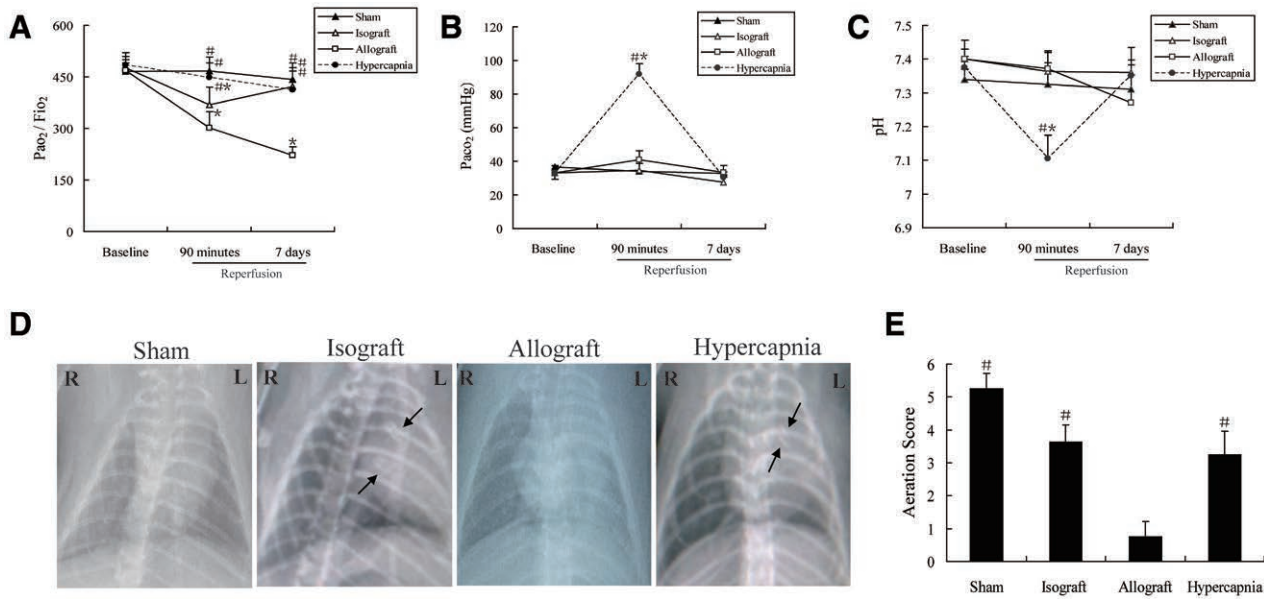


Fig. 1. Hypercapnia therapy protected pulmonary function. Arterial blood gas analysis at baseline, after reperfusion of 90 min, and on day 7. PaO₂/FiO₂ (A), PaCO₂ (B), and pH (C) in sham-operated, isograft, allograft, and hypercapnia groups. Representative x-ray of lung grafts (D) and aeration scores (E) in sham-operated, isograft, allograft, and hypercapnia groups. “Cuff-links” sign was apparent in the grafts (arrows in D). For A–C, we performed a two-way repeated measures ANOVA with time as a within-subject factor and group as a between-subject factor. We used the Student–Newman–Keuls *post hoc* test to perform multigroup comparisons for the values (PaO₂/FiO₂, PaCO₂, and pH) at different time points (baseline, at 90 min, and 7 days after reperfusion); moreover, in each group, we used the Student–Newman–Keuls *post hoc* test to perform repeated comparisons for the values (PaO₂/FiO₂, PaCO₂, and pH) at the time points of 90 min (or 7 days) after reperfusion against the baseline. For E, multigroup comparisons were performed with one-way ANOVA followed by the Student–Newman–Keuls test. **P* < 0.05 versus allograft group. **P* < 0.05 versus baseline. L = left; R = right.

Table 1. Wet Weight-to-Dry Weight Ratio and Serum Levels of Inflammatory Cytokines

Groups	Wet Weight-to-Dry Weight Ratio	Tumor Necrosis Factor- α , pg/ml	Interleukin-2, pg/ml	Interleukin-10, pg/ml	Interferon- γ , pg/ml
Sham	2.40 \pm 0.43*	21 \pm 4*	2.1 \pm 0.9*	2.9 \pm 0.7*	4.8 \pm 1.9*
Isograft	3.43 \pm 0.50*	219 \pm 35*	28.8 \pm 7.3*	15.8 \pm 3.7	22.7 \pm 6.3*
Allograft	7.04 \pm 0.80	292 \pm 49	52.7 \pm 8.3	17.9 \pm 7.1	62.7 \pm 10.1
Hypercapnia	4.23 \pm 0.54*	208 \pm 40*	30.6 \pm 6.7*	20.4 \pm 6.8	28.1 \pm 4.9*

Differences of wet weight-to-dry weight ratio (or levels of inflammatory cytokines) among groups were analyzed by using one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test.

**P* < 0.05 versus allograft group.

The lung grafts in the sham-operated and isograft groups exhibited a relatively normal histology with no apparent morphologic changes (fig. 5A). The grafts in the allograft group exhibited severe edema in the alveolar septa and spaces, extensive leukocyte infiltration, intraalveolar hemorrhage, and thickening and breaking of alveolar walls. In the hypercapnia group, grafts exhibited a relatively intact alveolar structure, less extensive leukocyte infiltration, and thin and intact alveolar walls. Rejection scores in the hypercapnia groups were significantly lower than that in the allograft group (2 \pm 1 *vs.* 4 \pm 1, *P* = 0.021; fig. 5B).

Serum concentrations of tumor necrosis factor- α , interleukin-2, and interferon- γ in the sham-operated (21 \pm 4 *vs.* 292 \pm 49, 2.1 \pm 0.9 *vs.* 52.7 \pm 8.3, and 4.8 \pm 1.9 *vs.* 62.7 \pm 10.1,

P < 0.0001 for all), hypercapnia (208 \pm 40 *vs.* 292 \pm 49, 30.6 \pm 6.7 *vs.* 52.7 \pm 8.3, and 28.1 \pm 4.9 *vs.* 62.7 \pm 10.1; *P* = 0.029, *P* = 0.015, *P* < 0.0001, respectively), and isograft (219 \pm 35 *vs.* 292 \pm 49, 28.8 \pm 7.3 *vs.* 52.7 \pm 8.3, and 22.7 \pm 6.3 *vs.* 62.7 \pm 10.1, *P* = 0.035, *P* = 0.008, *P* < 0.0001, respectively) groups were lower than those in the allograft group (table 1). Hypercapnia (20.4 \pm 6.8 *vs.* 17.9 \pm 7.1, *P* = 0.093) and isograft (15.8 \pm 3.7 *vs.* 17.9 \pm 7.1, *P* = 0.244) groups exhibited similar interleukin-10 levels compared to the allograft group.

No severe injury to critical organs was detected in the sham-operated and isograft groups (fig. 6). In the allograft group, the native (right) lung showed local monocytic infiltration and wider alveolar septa; the liver revealed cellular

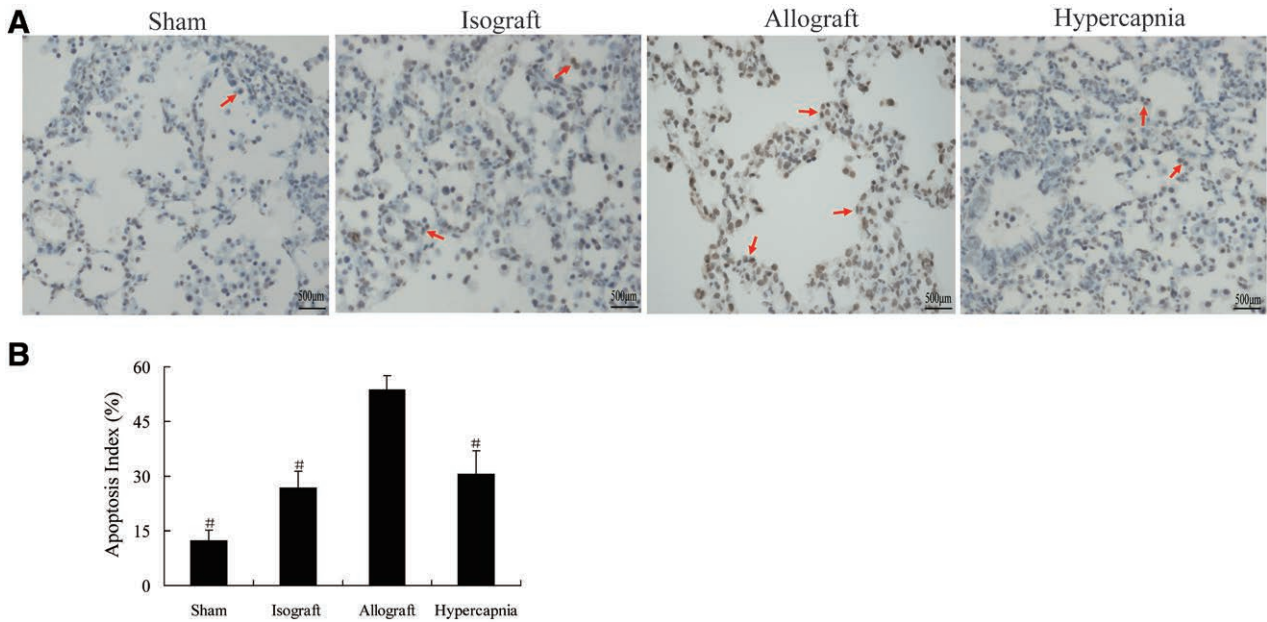


Fig. 2. Hypercapnia therapy decreased apoptosis in the lung grafts. Representative lung graft TdT-mediated dUTP nick end labeling staining (200 \times ; A) and apoptosis index (B) in sham-operated, isograft, allograft, and hypercapnia groups. Apoptosis of the cell was represented by brown-yellow staining in the nuclei (arrows in A). Differences in apoptosis indices among groups were analyzed by one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test. $\#P < 0.05$ versus allograft group.

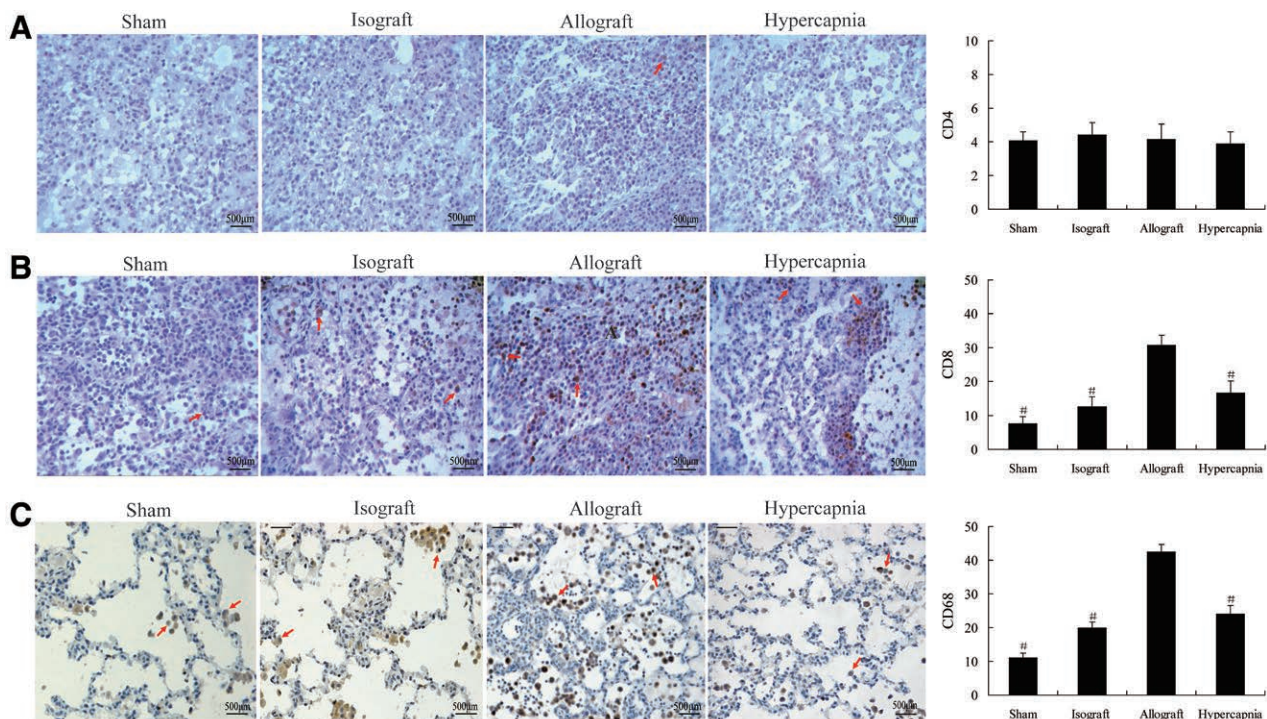


Fig. 3. Hypercapnia therapy alleviated the infiltration of T lymphocytes and macrophages in the lung grafts. Representative lung graft immunohistochemistry and statistical charts of immunohistochemical staining for CD4 (A), CD8 (B), and CD68 (C) in sham-operated, isograft, allograft, and hypercapnia groups (200 \times). The presence of brown granules in the cytoplasm indicated a positive stain (arrows in A–C). Differences of CD4, CD8, and CD68 among groups were analyzed using one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test. $\#P < 0.05$ versus allograft group.

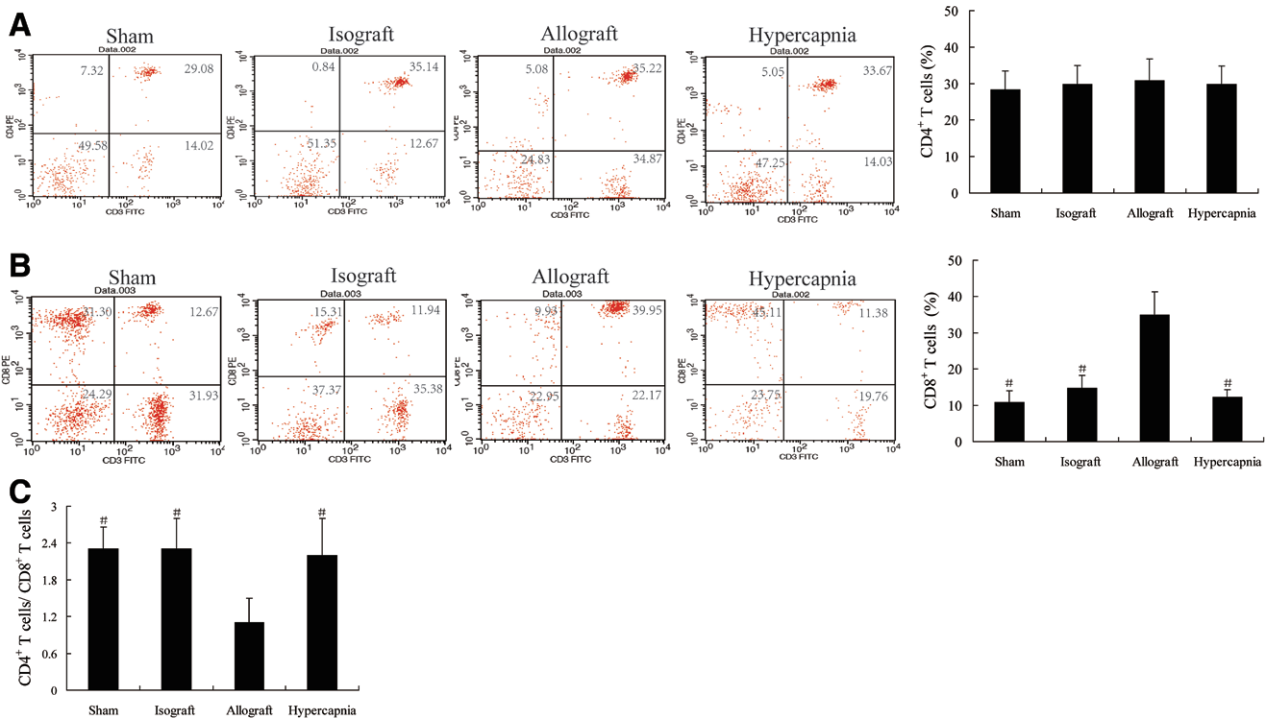


Fig. 4. Hypercapnia therapy alleviated the accumulation of T lymphocytes in the peripheral blood. Representative fluorescence-activated cell sorting scatterplots and statistical charts of CD4⁺ T cells (A), CD8⁺ T cells (B), and CD4⁺/CD8⁺ T cell ratio (C) in sham-operated, isograft, allograft, and hypercapnia groups. Difference of T cell subsets and CD4⁺/CD8⁺ T cell ratio among groups were analyzed by using one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test. #*P* < 0.05 versus allograft group.

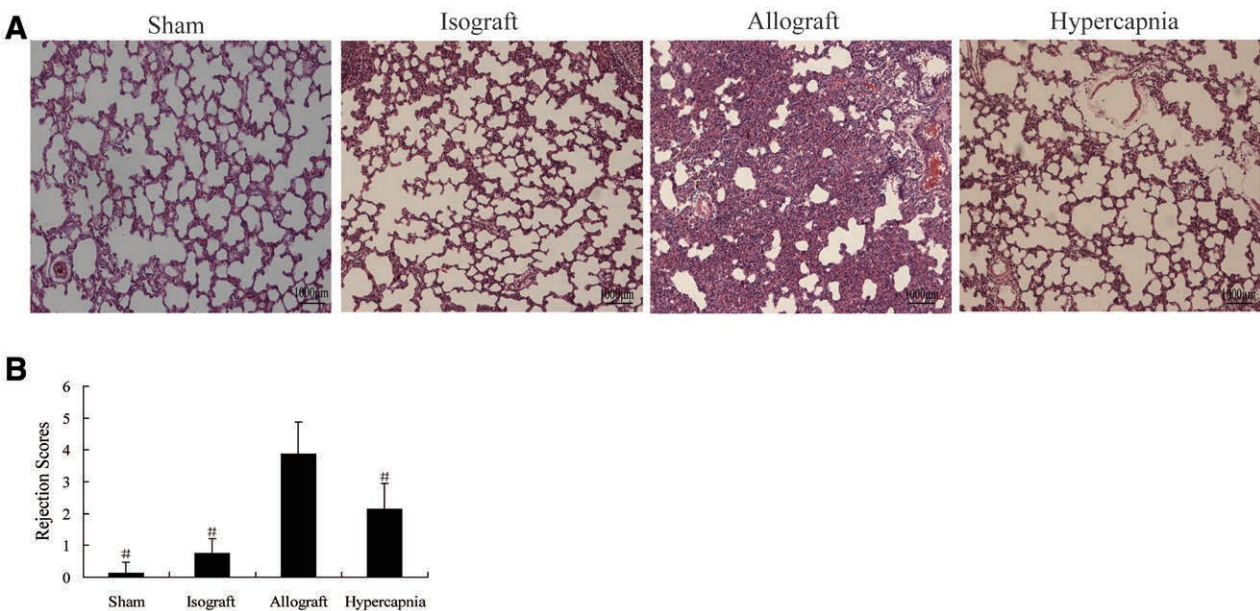


Fig. 5. Hypercapnia decreased rejection scores in the lung grafts. Hematoxylin and eosin staining of representative lung graft (100x; A) and rejection scores (B) in sham-operated, isograft, allograft, and hypercapnia groups. Difference of rejection scores among groups were analyzed by using one-way ANOVA followed by the Student–Newman–Keuls *post hoc* test. #*P* < 0.05 versus allograft group.

edema, congestion, and vacuolar degeneration; and cardiac muscle displayed irregularities and congestion. Hypercapnia therapy partly alleviated these morphologic changes. No obvious injuries in the brain were observed in any of the groups.

Discussion

Hypercapnia augments physiologic functioning in allograft recipients as it increases hemoglobin concentration and elevates arterial oxygen content.²⁴ Deliberate elevation of P_{aCO_2} protects against pulmonary ischemia–reperfusion injury, and thus, the use of hypercapnia may have a theoretical rationale in the prevention and treatment of acute cellular rejection. In our study, the hypercapnia group showed higher P_{aO_2}/F_{IO_2} and aeration scores and lower wet weight-to-dry weight ratio and apoptosis as compared to that in the allograft group, which indicates that hypercapnia can enhance the oxygen-carrying capacity and improve lung graft function. Moreover, hypercapnia reduced perivascular and peribronchial monocyte infiltration, alleviated destruction of alveolar wall

structure, and ultimately lowered pulmonary parenchyma damage and rejection scores.

Preliminary experiments demonstrated that inspired carbon dioxide fraction ranging from 0 to 15% during reperfusion increased P_{aO_2}/F_{IO_2} and reduced wet weight-to-dry weight ratio in a dose-dependent manner. However, the magnitude of decrease in wet weight-to-dry weight diminished at inspired carbon dioxide of more than 8%. Moreover, exposure of recipients to 12 to 15% CO_2 (P_{aCO_2} 120 to 140 mmHg) led to hemodynamic instability marked by decreased mean arterial pressure. Protective effects of hypercapnia may be offset by potential adverse effects at higher levels of inspired carbon dioxide, thus giving rise to an “effect ceiling.”²⁵ Inhalation of 8% CO_2 at a P_{aCO_2} in the range 80 to 100 mmHg was safe and effective. We selected carbon dioxide inhalation for 90 min during reperfusion based on a fundamental study by Laffey *et al.*⁸ on the effect of hypercapnia in reducing pulmonary ischemia–reperfusion injury. Moreover, considering the benefit of long-term survival after transplantation, we selected a relevant short reperfusion to

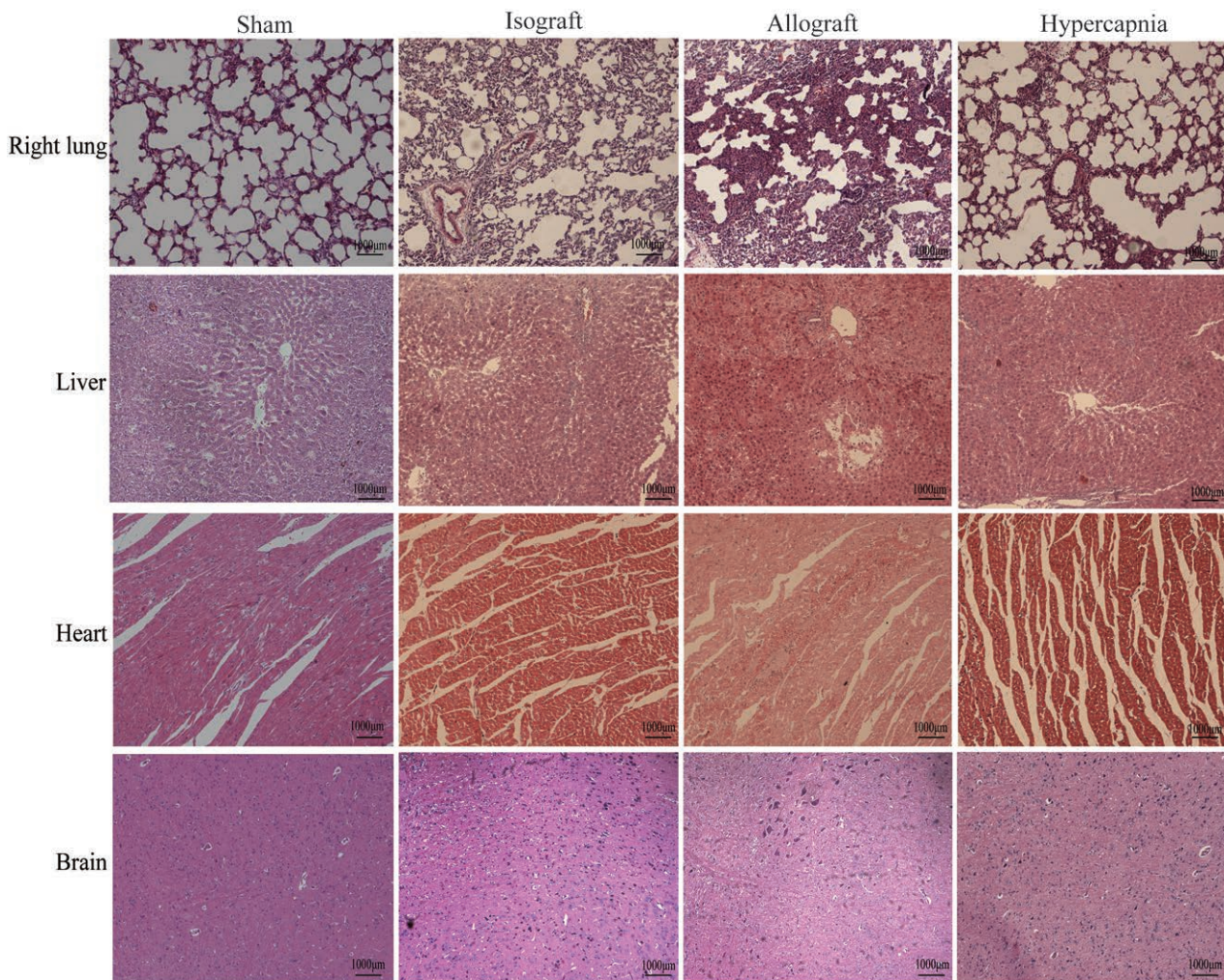


Fig. 6. Hypercapnia decreased multiple organ injuries. Hematoxylin- and eosin-stained sections of right lung, liver, heart, and brain in sham-operated, isograft, allograft, and hypercapnia groups (100×) are shown.

minimize ventilator-induced lung injury. A rapid decrease in P_{aCO_2} from 100 mmHg (reperfusion endpoint) to 58 mmHg (well spontaneous breath) was observed within 30 min. Another 60 min were required to lower the P_{aCO_2} values to the normal level. The actual duration of hypercapnia was approximately 3 h in the present study.

Apoptosis after lung transplantation results in the loss of lung function. Our recent studies showed that reperfusion with blood recirculation leads to a rapid elevation in pH, which modulates apoptosis.²⁶ In the present study, pH decreased after reperfusion in the hypercapnia group, which exhibited a lower apoptosis index. Hypercapnia can up-regulate some antiapoptotic proteins, such as Bcl-2 and Bcl-xL, and reduce lung apoptosis to protect from pulmonary reperfusion injury.^{27,28} Hypercapnia inhibits the “apoptosis burst” after early reperfusion and may subsequently prevent initiation of apoptotic cascade for several days after lung transplantation.

T cell-regulated inflammatory response participates in mechanisms of acute rejection, including T cell apoptosis and anergy-induced immune tolerance. Carbon dioxide insufflation depresses the functioning of peritoneal macrophages, neutrophils, and T lymphocytes and causes systemic immune disturbances.^{29,30} Such local immune depression persists for at least 7 days.³¹ In our study, the hypercapnia group had lower CD8 and CD68 staining in grafts, which suggests that reduction in CD8⁺ T cells and CD68⁺ macrophages may alleviate the immune response. Activated CD4⁺ T cells release cytokines that promote proliferation and maturation of CD8⁺ T cells, whereas CD8⁺ T cell-dependent cytotoxicity directly kills target cells. In our study, hypercapnia reduced CD8 in grafts and CD8⁺ T cell counts in peripheral blood, which indicates that hypercapnia suppresses the infiltration and accumulation of CD8⁺ T cells.

Between-group differences with respect to CD4 staining in grafts and CD4⁺ T cell counts in peripheral blood were not statistically significant. This may indicate that CD8⁺ T cells predominated over CD4⁺ T cells in pulmonary acute cellular rejection in our experiment. However, this was assessed only at a single time point (postoperative day 7), which is a limitation. The results may have been different if we had selected more determination points, such as at 2 weeks or 1 month after transplantation. A lower CD4/CD8 ratio has been associated with a higher possibility of lung allograft rejection in the first year after transplantation.³² The CD4/CD8 ratio increased in the hypercapnia group, which suggests that hypercapnia may modulate T lymphocyte responses and help decrease the chances of acute cellular rejection. Insufflation of carbon dioxide for 30 min in rat laparoscopy models suppressed immune response secondary to a decrease in lymphocyte and macrophage counts and tumor necrosis factor- α level at 1 h after intervention.³³ Our results support these findings in a rat model of acute lung rejection over a longer period (7 days).

Cytokines participate in acute rejection or tolerance. Upon activation of CD4⁺ T cells, numerous cytokines are released by subsets of helper T cells (Th): Th1 and Th2. Further, strong expression of Th1 cytokines tumor necrosis factor- α , interleukin-2, and interferon- γ and mild suppression of Th2-cytokines interleukin-4, interleukin-6, and interleukin-10 are associated with early rejection.³⁴ Several studies have shown that Th1 cytokines play a more dominant role than Th2 cytokines in the cellular immune response during acute rejection of lung and other solid organs,^{34–36} which was also supported by our findings. Our results showed that hypercapnia significantly reduced plasma concentrations of interleukin-2, tumor necrosis factor- α , and interferon- γ . Hypercapnia decreased the levels of CD4⁺ T cell-derived cytokines without having any influence on the CD4⁺ T cell counts, which indicates reduced CD4⁺ T cell activity. Although elevated levels of interleukin-10 may induce immune tolerance to lung transplants,³⁷ this phenomenon was not observed in the present study.

Acute cellular rejection represents a systemic inflammatory state that induces multisystem injuries. Hypercapnia was shown to reduce pulmonary and systemic injury after lung ischemia–reperfusion through attenuation of the levels of systemic cytokines and free radicals.⁸ In our study, hematoxylin and eosin-stained sections of right lung, liver, and heart tissues in the hypercapnia group showed alleviation of injury. Increased expression of potassium–adenosine triphosphate channels is involved in mediating the protective effect of hypercapnia against secondary visceral organ dysfunction.³⁸ We did not observe any signs of brain tissue damage in all the study groups; this is ostensibly due to the protective effect of endothelial cells comprising the blood–brain barrier, which may have prevented the entry of activated Th1 cells into brain tissue.³⁹

Lung-protective ventilation (with low V_T and high PEEP) improves survival in patients with ARDS.⁴⁰ A recent study by Hummler *et al.*⁴¹ showed that the therapeutic effect of hypercapnia induced by protective ventilation (V_T 4.5 ml/kg and PEEP 7 cm H₂O) was superior to that of hypercapnia alone (V_T 7.5 ml/kg, PEEP 7 cm H₂O and inhalation of 4% CO₂) in a rabbit model of ARDS. However, ventilation settings used for hypercapnia alone in their study are still in the lung-protective ventilation category. In our experiment, ventilation settings (V_T = 8 to 10 ml/kg, PEEP = 2 cm H₂O) were not protective in all groups. This implies that the protective effect observed in the hypercapnia group occurred in the context of ordinary ventilation model, which served to distinguish and elicit the protective effect of hypercapnia alone and excluded the effect of low V_T -induced decreased ventilator-induced lung injury.

Several considerations about future clinical application of hypercapnia are noteworthy. First, we did not administer any other immunomodulating agents in the present study, and we aimed to explore the sole protective effect of hypercapnia in a severe acute cellular rejection setting. This is inconsistent

with clinical rejection treatment, because antirejection medication constitutes an integral component of modern transplant programs. We presume that hypercapnia may have an additive and independent immunomodulatory effect when used together with other immunosuppressor agents. Second, the immunosuppressive effect of severe and sustained hypercapnia impairs the host response to bacterial invasion and increases lung infection. Protective strategies of minimizing the duration and dose of inhaled carbon dioxide in conjunction with early effective antibiotic therapy may confer an advantage.^{42,43} Of note, infections resulting from short carbon dioxide inhalation have not yet been documented. Third, hypercapnia increases pulmonary artery pressure (Ppa) and right-heart strain. This seems to be a paradox in hypercapnia treatment for acute cellular rejection in recipients who have developed right heart dysfunction. Laffey *et al.*^{25,44} investigated Ppa after short inhalation of 5% or 25% CO₂ in animal models. They reported a maximum Ppa of less than 24 mmHg and gentle elevation of Ppa by 2 mmHg, which does not qualify the criteria for pulmonary hypertension (mean Ppa \geq 25 mmHg at rest).⁴⁵ These data may provide a valuable reference for future clinical hypercapnia application. In addition, alleviation of acute cellular rejection protects against immune-mediated microvascular endothelial cell injury, decreases the severity of vasculitis, and inhibits pulmonary arterial fibrous stenosis,⁴⁶ which may help maintain normal pulmonary vascular pressure after hypercapnia treatment. Safety remains a key factor for a possible transposition from an animal study to a clinical one. Luckily, hypercapnia did not induce complications as mentioned in all groups, such as obvious lung infection (evidenced by rat x-rays) and high Ppa (inferring from Laffey *et al.*'s data). However, complications should be closely observed in further clinical transformation, with an intent to institute aggressive prevention strategies.

In summary, hypercapnia effectively protects lung function and ameliorates acute cellular rejection and injury to other critical organs after lung transplantation in a rat model by inhibiting the activation and accumulation of T cells and macrophages, suppressing release of proinflammatory cytokines, and inhibiting apoptosis. This provides a theoretical basis for the development of novel strategies against acute cellular rejection after lung transplantation.

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Competing Interests

The authors declare no competing interests.

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Address correspondence to Dr. Cui: Second Affiliated Hospital of Harbin Medical University, No. 246, XueFu Road, Nan-Gang District, Harbin 150081, Heilongjiang Province, China. cuixiaoguang1018@126.com. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

References

- Christie JD, Edwards LB, Kucheryavaya AY, Benden C, Dobbels F, Kirk R, Rahmel AO, Stehlik J, Hertz MI: The Registry of the International Society for Heart and Lung Transplantation: Twenty-eighth Adult Lung and Heart-Lung Transplant Report—2011. *J Heart Lung Transplant* 2011; 30:1104–22
- Moreau A, Valey E, Anegon I, Cuturi MC: Effector mechanisms of rejection. *Cold Spring Harb Perspect Med* 2013; 3:113–9
- Jungraithmayr W, Inci I, Bain M, Hillinger S, Korom S, Weder W: Distribution of macrophages and T cells in syngrafts and allografts after experimental rat lung transplantation. *Immunobiology* 2010; 215:206–14
- Hodge G, Hodge S, Chambers DC, Reynolds PN, Holmes M: Increased expression of graft intraepithelial T-cell pro-inflammatory cytokines compared with native lung during episodes of acute rejection. *J Heart Lung Transplant* 2012; 31:538–44
- Geleff S, Draganovici D, Jaksch P, Segerer S: The role of chemokine receptors in acute lung allograft rejection. *Eur Respir J* 2010; 35:167–75
- Merry HE, Phelan P, Doak MR, Zhao M, Hwang B, Mulligan MS: Role of Toll-like receptor-4 in lung ischemia-reperfusion injury. *Ann Thorac Surg* 2015; 99:1193–9
- Gao W, Liu DD, Li D, Cui GX: Effect of therapeutic hypercapnia on inflammatory responses to one-lung ventilation in lobectomy patients. *ANESTHESIOLOGY* 2015; 122:1235–52
- Laffey JG, Tanaka M, Engelberts D, Luo X, Yuan S, Tanswell AK, Post M, Lindsay T, Kavanagh BP: Therapeutic hypercapnia reduces pulmonary and systemic injury following *in vivo* lung reperfusion. *Am J Respir Crit Care Med* 2000; 162:2287–94
- Nichol AD, O'Cronin DE, Naughton F, Hopkins N, Boylan J, McLoughlin P: Hypercapnic acidosis reduces oxidative reactions in endotoxin-induced lung injury. *ANESTHESIOLOGY* 2010; 113:116–25
- Contreras M, Ansari B, Curley G, Higgins BD, Hassett P, O'Toole D, Laffey JG: Hypercapnic acidosis attenuates ventilation-induced lung injury by a nuclear factor- κ B-dependent mechanism. *Crit Care Med* 2012; 40:2622–30
- Masterson C, Otulakowski G, Kavanagh BP: Hypercapnia: Clinical relevance and mechanisms of action. *Curr Opin Crit Care* 2015; 21:7–12
- Sewing AC, Kantores C, Ivanovska J, Lee AH, Masood A, Jain A, McNamara PJ, Tanswell AK, Jankov RP: Therapeutic hypercapnia prevents bleomycin-induced pulmonary hypertension in neonatal rats by limiting macrophage-derived tumor necrosis factor- α . *Am J Physiol Lung Cell Mol Physiol* 2012; 303:L75–87
- Zhou Q, Cao B, Niu L, Cui X, Yu H, Liu J, Li H, Li W: Effects of permissive hypercapnia on transient global cerebral ischemia-reperfusion injury in rats. *ANESTHESIOLOGY* 2010; 112:288–97
- Li AM, Quan Y, Guo YP, Li WZ, Cui XG: Effects of therapeutic hypercapnia on inflammation and apoptosis after hepatic ischemia-reperfusion injury in rats. *Chin Med J (Engl)* 2010; 123:2254–8

15. Wang N, Gates KL, Trejo H, Favoreto S Jr, Schleimer RP, Sznajder JI, Beitel GJ, Sporn PH: Elevated CO₂ selectively inhibits interleukin-6 and tumor necrosis factor expression and decreases phagocytosis in the macrophage. *FASEB J* 2010; 24:2178–90
16. Hamad M, Irhimeh MR, Abbas A: Hypercapnia slows down proliferation and apoptosis of human bone marrow promyeloblasts. *Bioprocess Biosyst Eng* 2016; 39:1465–75
17. Robertson CE, Turko AJ, Jonz MG, Wright PA: Hypercapnia and low pH induce neuroepithelial cell proliferation and emersion behaviour in the amphibious fish *Kryptolebias marmoratus*. *J Exp Biol* 2015; 218:2987–90
18. Gao W, Liu D, Li D, Che X, Cui G: Effects of hypercapnia on T cells in lung ischemia/reperfusion injury after lung transplantation. *Exp Biol Med (Maywood)* 2014; 239:1597–605
19. Lv X, Tan J, Liu D, Wu P, Cui X: Intratracheal administration of p38 α short-hairpin RNA plasmid ameliorates lung ischemia-reperfusion injury in rats. *J Heart Lung Transplant* 2012; 31:655–62
20. Prop J, Wagenaar-Hilbers JP, Petersen AH, Wildevuur CR: Diagnosis of rejection in rat lung allografts by bronchoalveolar lavage. *Transplant Proc* 1987; 19:3779–80
21. Hirayama S, Shiraishi T, Shirakusa T, Higuchi T, Miller EJ: Prevention of neutrophil migration ameliorates rat lung allograft rejection. *Mol Med* 2006; 12:208–13
22. Zhou H, Fu Z, Wei Y, Liu J, Cui X, Yang W, Ding W, Pan P, Li W: Hydrogen inhalation decreases lung graft injury in brain-dead donor rats. *J Heart Lung Transplant* 2013; 32:251–8
23. Zhou H, Qian H, Liu J, Zhu D, Ding W, Pan P, Jin D, Wang J, Li W: Protection against lung graft injury from brain-dead donors with carbon monoxide, biliverdin, or both. *J Heart Lung Transplant* 2011; 30:460–6
24. Li J, Zhang G, Holtby H, Bissonnette B, Wang G, Redington AN, Van Arsdell GS: Carbon dioxide—a complex gas in a complex circulation: Its effects on systemic hemodynamics and oxygen transport, cerebral, and splanchnic circulation in neonates after the Norwood procedure. *J Thorac Cardiovasc Surg* 2008; 136:1207–14
25. Laffey JG, Jankov RP, Engelberts D, Tanswell AK, Post M, Lindsay T, Mullen JB, Romaschin A, Stephens D, McKelvie C, Kavanagh BP: Effects of therapeutic hypercapnia on mesenteric ischemia-reperfusion injury. *Am J Respir Crit Care Med* 2003; 168:1383–90
26. Tan J, Liu D, Lv X, Wang L, Zhao C, Che Y, Xie Q, Cui X: MAPK mediates inflammatory response and cell death in rat pulmonary microvascular endothelial cells in an ischemia-reperfusion model of lung transplantation. *J Heart Lung Transplant* 2013; 32:823–31
27. Casalino-Matsuda SM, Nair A, Beitel GJ, Gates KL, Sporn PH: Hypercapnia inhibits autophagy and bacterial killing in human macrophages by increasing expression of Bcl-2 and Bcl-xL. *J Immunol* 2015; 194:5388–96
28. Wu SY, Li MH, Ko FC, Wu GC, Huang KL, Chu SJ: Protective effect of hypercapnic acidosis in ischemia-reperfusion lung injury is attributable to upregulation of heme oxygenase-1. *PLoS One* 2013; 8:e74742
29. Chen YZ, Xu YF, Zheng JH: Effect of carbon dioxide pneumoperitoneum on human renal cell carcinoma proliferation and metastasis in an orthotopic xenograft nude mouse model. *Arch Med Sci* 2014; 10:1041–6
30. Brokelman WJ, Lensvelt M, Borel Rinkes IH, Klinkenbijl JH, Reijnen MM: Peritoneal changes due to laparoscopic surgery. *Surg Endosc* 2011; 25:1–9
31. Neuhaus SJ, Watson DI, Ellis T, Rofo AM, Mathew G, Jamieson GG: Influence of gases on intraperitoneal immunity during laparoscopy in tumor-bearing rats. *World J Surg* 2000; 24:1227–31
32. Shenoy KV, Solomides C, Cordova F, Rogers TJ, Ciccolella D, Criner GJ: Low CD4/CD8 ratio in bronchus-associated lymphoid tissue is associated with lung allograft rejection. *J Transplant* 2012; 2012:928081
33. Jacobi CA, Wenger F, Sabat R, Volk T, Ordemann J, Müller JM: The impact of laparoscopy with carbon dioxide *versus* helium on immunologic function and tumor growth in a rat model. *Dig Surg* 1998; 15:110–6
34. Lemaître PH, Vokaer B, Charbonnier LM, Iwakura Y, Field KA, Estenne M, Goldman M, Leo O, Rummelink M, Le Moine A: Cyclosporine A drives a Th17- and Th2-mediated posttransplant obliterative airway disease. *Am J Transplant* 2013; 13:611–20
35. van der Leij J, van den Berg A, Albrecht EW, Blokzijl T, Roozendaal R, Gouw AS, de Jong KP, Stegeman CA, van Goor H, Chang NS, Poppema S: High expression of TIAF-1 in chronic kidney and liver allograft rejection and in activated T-helper cells. *Transplantation* 2003; 75:2076–82
36. Nakagiri T, Warnecke G, Avsar M, Thissen S, Kruse B, Kühn C, Ziehme P, Knöfel AK, Madrahimov N, Okumura M, Sawa Y, Gottlieb J, Simon AR, Haverich A, Strüder M: Lung function early after lung transplantation is correlated with the frequency of regulatory T cells. *Surg Today* 2012; 42:250–8
37. Zhou W, Zhou X, Gaowa S, Meng Q, Zhan Z, Liu J, Li J, Fan H, Liu Z: The critical role of induced CD4+ FoxP3+ regulatory cells in suppression of interleukin-17 production and attenuation of mouse orthotopic lung allograft rejection. *Transplantation* 2015; 99:1356–64
38. Najarian T, Marrache AM, Dumont I, Hardy P, Beauchamp MH, Hou X, Peri K, Gobeil F Jr, Varma DR, Chemtob S: Prolonged hypercapnia-evoked cerebral hyperemia via K⁺ channel- and prostaglandin E₂-dependent endothelial nitric oxide synthase induction. *Circ Res* 2000; 87:1149–56
39. Biernacki K, Prat A, Blain M, Antel JP: Regulation of Th1 and Th2 lymphocyte migration by human adult brain endothelial cells. *J Neuropathol Exp Neurol* 2001; 60:1127–36
40. Amato MB, Barbas CS, Medeiros DM, Magaldi RB, Schettino GP, Lorenzi-Filho G, Kairalla RA, Deheinzelin D, Munoz C, Oliveira R, Takagaki TY, Carvalho CR: Effect of a protective-ventilation strategy on mortality in the acute respiratory distress syndrome. *N Engl J Med* 1998; 338:347–54
41. Hummler HD, Banke K, Wolfson MR, Buonocore G, Ebsen M, Bernhard W, Tsikas D, Fuchs H: The effects of lung protective ventilation or hypercapnic acidosis on gas exchange and lung injury in surfactant deficient rabbits. *PLoS One* 2016; 11:e0147807
42. O’Croinin DF, Nichol AD, Hopkins N, Boylan J, O’Brien S, O’Connor C, Laffey JG, McLoughlin P: Sustained hypercapnic acidosis during pulmonary infection increases bacterial load and worsens lung injury. *Crit Care Med* 2008; 36:2128–35
43. Chonghaile MN, Higgins BD, Costello J, Laffey JG: Hypercapnic acidosis attenuates lung injury induced by established bacterial pneumonia. *ANESTHESIOLOGY* 2008; 109:837–48
44. Laffey JG, Engelberts D, Kavanagh BP: Buffering hypercapnic acidosis worsens acute lung injury. *Am J Respir Crit Care Med* 2000; 161:141–6
45. Chen W, Li S: Circulating microRNA as a novel biomarker for pulmonary arterial hypertension due to congenital heart disease. *Pediatr Cardiol* 2017; 38:86–94
46. Kim HK, Kim JE, Wi HC, Lee SW, Kim JY, Kang HJ, Kim YT: Aurintricarboxylic acid inhibits endothelial activation, complement activation, and von Willebrand factor secretion *in vitro* and attenuates hyperacute rejection in an *ex vivo* model of pig-to-human pulmonary xenotransplantation. *Xenotransplantation* 2008; 15:246–56