Unilateral Acid Aspiration Augments the Effects of Ventilator Lung Injury in the Contralateral Lung

Maria Amigoni, M.D.,* Giacomo Bellani, M.D., Ph.D.,† Vanessa Zambelli, Biol.D.,‡ Margherita Scanziani,M.D.,§ Francesca Farina, Biol.D,. Ph.D.,‡ Lorella Fagnani, M.D.,§ Roberto Latini, M.D.,|| Roberto Fumagalli, M.D.,# Antonio Pesenti, M.D.**

ABSTRACT

Background: Mechanical ventilation is necessary during acute respiratory distress syndrome, but it promotes lung injury because of the excessive stretch applied to the aerated parenchyma. The authors' hypothesis was that after a regional lung injury, the noxious effect of mechanical ventilation on the remaining aerated parenchyma would be more pronounced.

Methods: Mice, instilled with hydrochloric acid (HCl) in the right lung, was assigned to one of the following groups: mechanical ventilation with tidal volumes (V_T) 25 ml/kg (HCl-VILI$_{25}$, n = 12), or V_T 15 ml/kg (HCl-VILI$_{15}$, n = 9), or spontaneous breathing (HCl-SB, n = 14). Healthy mice were ventilated with V_T 25 ml/kg (VILI$_{25}$, n = 11). Arterial oxygenation, lung compliance, bronchoalveolar lavage inflammatory cells, albumin, and cytokines concentration were measured.

Results: After 7 h, oxygenation and lung compliance resulted lower in HCl-VILI$_{25}$ than in VILI$_{25}$ ($P < 0.05$, 210 ± 54 *vs.* 479 ± 83 mmHg, and 32 ± 3.5 *vs.* 45 ± 4.1 μl/cm H_2O, mean ± SD, respectively). After right lung injury, the left lung of HCl-VILI$_{25}$ group received a greater fraction of the V_T than the VILI$_{25}$ group, despite an identical global V_T. The number of total and polymorphonuclear cells in bronchoalveolar lavage resulted significantly higher in HCl-VILI$_{25}$, compared with the other groups, in not only the right lung, but also in the left lung. The albumin content in the left lung resulted higher in HCl-VILI$_{25}$ than in VILI$_{25}$ (224 ± 85 *vs.* 33 ± 6 μg/ml; $P < 0.05$). Cytokines levels did not differ between groups.

Conclusion: Aggressive mechanical ventilation aggravates the preexisting lung injury, which is noxious for the contralateral, not previously injured lung, possibly because of a regional redistribution of V_T.

What We Already Know about This Topic

- Ventilator-induced lung injury occurs, in part, by overstretching nearly normal lung approximate to injured lung

What This Article Tells Us That Is New

- A unilateral loss of aeration can lead to ventilator-induced lung injury in the contralateral uninjured lung

IN acute respiratory failure, mechanical ventilation (MV) may be a necessary life-saving treatment. MV, as is largely reported, can induce lung injury in the healthy lung or exacerbate a preexisting lung injury. Minimization of ventilator-induced lung injury (VILI) by reduction of tidal volumes (V_T) from 12 to 6 ml/kg significantly improved the outcome of acute respiratory distress syndrome patients.[1] However, MV may evoke VILI, particularly in previously injured lungs, even if low V_T are used.[2–4] Several studies demonstrated that the injured lung is largely susceptible to the effects of overdistension[5,6] or "alveolar opening and collapse" induced by MV.[7–9] VILI is characterized by a pulmonary inflammatory response with a local release of cytokines, recruitment of leukocytes in the lung, and increased lung permeability, resulting in lung edema, surfactant dysfunction, impaired lung compliance, and deterioration of pulmonary gas exchange[3–13]. Moreover, as previously

* Staff Physician, Department of Emergency, San Gerardo Hospital, Monza (MB), Italy. † Staff Physician, Department of Emergency, San Gerardo Hospital, and Researcher, Department of Health Science, University of Milano-Bicocca, Monza (MB), Italy. ‡ Research Fellow, § Resident, # Associate Professor of Anesthesia, Department of Health Science, University of Milano-Bicocca. || Research Scientist, Department of Cardiovascular Research, Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy. ** Professor of Anesthesia, Department of Health Science, University of Milano-Bicocca, and Director, Department of Emergency, San Gerardo Hospital.

Received from the Department of Experimental Medicine (DIMS), University of Milano-Bicocca, Monza (MB), Italy. Submitted for publication February 14, 2012. Accepted for publication April 12, 2013. This study was partially supported by Chiesi Farmaceutici, Parma, Italy; and by departmental funding. This work has been presented in part at the 24th Annual Congress of the European Society of Intensive Care Medicine, October 1–5, 2011, Berlin, Germany.

Address correspondence to Dr. Bellani: Department of Experimental Medicine (DIMS), University of Milano-Bicocca, Via Cadore 48, 20900 Monza, MB, Italy. giacomo.bellani1@unimib.it. Anesthesiology 2013; 119:642-51

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are available in both the HTML and PDF versions of this article. Links to the digital files are provided in the HTML text of this article on the Journal's Web site (www.anesthesiology.org).

described,[14,15] injurious ventilation strategies eliciting the release of proinflammatory mediators from the lung may lead to an increase in systemic cytokines concentration, which may initiate or propagate a multisystem organ failure.

Most of the models of VILI reported in the literature investigated the effects of MV with high inspiratory peak pressure (above 30 cm H_2O) and very high V_T (above 30 ml/kg).[10,12,16,17] Conversely, we attempted to simulate a clinical situation during which one part of the lung is excluded from ventilation as a consequence of the "primary" acute respiratory distress syndrome process, but the whole respiratory system is subject to MV (adjusted to achieve peak inspiratory pressure not exceeding 25 cm H_2O). For this purpose we set up a "two-hit" lung injury model: unilateral acid instillation followed by MV prolonged for 7 h. The peculiarity of this model is the presence, in the same animal, of a lung (right), which is challenged by acid aspiration, and thus, less available for ventilation, whereas, the contralateral (left lung), not directly injured, is most likely subjected to a specific overventilation. In line with some literature data, suggesting that the ratio between end-expiratory lung volume and V_T is a major determinant of lung inflammation,[18–21] we hypothesized that the effect of VILI would have been more pronounced on the uninjured lung due to the reduced aeration of the injured lung.

Materials and Methods

Animals

Male C57/BL6J mice (22–25 g) were obtained from Harlan Laboratories (Udine, Italy) and maintained under standard laboratory condition at the University of Milano-Bicocca in Monza (Italy). Procedures involving animals and their care were conducted in conformity with the institutional guidelines, complying with national (D.L. n. 116, G.U., suppl. 40, 18 Febbraio 1992, Circolare n. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, December 12, 1987; *Guide for the Care and Use of Laboratory Animals*, U.S. National Research Council, 1996). The experimental protocol was submitted to the Italian Ministry of Health and approved by the Animal Care Unit of the University of Milan-Bicocca (Monza, Italy).

General Experimental Protocol

Fifteen minutes before induction, each animal received a subcutaneous ringer acetate bolus (300 μl) to enhance hemodynamic stability. Animals were anesthetized with ketamine (120 mg/kg), xylazine (0.8 mg/kg), and fentanyl (90 μg/kg) by intraperitoneal injection. They were then secured in supine position, orotracheally intubated with a 22-gauge catheter, and connected to a rodent ventilator (Inspira asv; Harvard Apparatus, Holliston, MA). The setting of MV differed among the experimental groups (see Experimental groups section). A PE-10 catheter was then introduced into the right bronchus through a small tracheal incision. Through this catheter, 1.5 ml/kg of 0.1 M hydrochloric acid (HCl) was instilled into the right lung (details were previously described).[22] To confirm the selectivity of HCl instillation, we studied eight mice that were instilled with methylene blue, by using the same procedure for HCl instillation, and underwent the low (15 ml/kg) and high (25 ml/kg) MV protocol (for details see Supplemental Digital Content 1, http://links.lww.com/ALN/A942, which contains a detailed description of the additional experiments made in order to verify the selectivity of acid instillation). Only during HCl instillation and the following 10 min, we set fraction of inspired oxygen (F_{IO_2}) to 1.0, to avoid hypoxia during the procedure. In the animals undergoing prolonged ventilation (see Experimental groups section), a PE-10 catheter was inserted in the arterial carotid, connected to a pressure transducer and to a syringe pump, for continuous liquid and anesthetics infusion. Neuromuscular blockade was obtained by an intraperitoneal injection of pancuronium bromide 2 mg/kg. The animals were placed on a heating pad and body temperature was maintained constant at about 36–37°C, for the entire duration of the experiment.

Experimental Groups

HCl-SB. After HCl instillation, animals were ventilated for 10 min, as (V_T 8 ml/kg, respiratory rate of 130 min^{-1}, positive end-expiratory pressure of 2-cm H_2O, F_{IO_2} of 1, inspiratory to expiratory ratio of 35%) and they were extubated after awakening, and put in an oxygenate chamber for 7 h.

HCl-$VILI_{15}$ Animals received the instillation of HCl, and then were mechanically ventilated for 7 h with a V_T of 15 ml/kg, respiratory rate of 130 min^{-1}, positive end-expiratory pressure of 2-cm H_2O, F_{IO_2} of 0.5, inspiratory to expiratory ratio of 35%.

HCl-$VILI_{25}$. Animals received the instillation of HCl, and then were mechanically ventilated for 7 h with a V_T of 25 ml/kg, respiratory rate of 100 min^{-1}, positive end-expiratory pressure of 2-cm H_2O, F_{IO_2} of 0.5, inspiratory to expiratory ratio of 35%.

$VILI_{25}$. Animals did not receive HCl instillation. They were only ventilated for 7 h with a V_T of 25 ml/kg, respiratory rate of 100 min^{-1}, positive end-expiratory pressure of 2-cm H_2O, F_{IO_2} of 0,5, inspiratory to expiratory ratio of 35%. To maintain P_{ACO_2} within a physiological range, a volume of dead space was added to ventilator circuit in this group of mice because preliminary experiments (data not shown) suggested that $VILI_{25}$ group tended to be severely hypocapnic after 30 min of ventilation with V_T of 25 ml/kg and respiratory rate of 100 min^{-1}.

Anesthesia Maintenance

An adequate level of anesthesia and hydration was maintained by the continuous infusion of fentanyl 2.4 $\mu g \cdot kg^{-1} \cdot h^{-1}$, ketamine 12 $mg \cdot kg^{-1} \cdot h^{-1}$, pancuronium bromide 0.3 $mg \cdot kg^{-1} \cdot h^{-1}$ in ringer acetate. The infusion rate was maintained at 14 μl/min for the first hour of MV, and 4 μl/min for the following hours; infusion rate was increased if hypotension developed.

Monitoring

During the experiment, hemodynamic parameters (arterial pressure and heart rate) and airway pressure were continuously monitored by using pressure transducers connected to a signal transduction unit (PowerLab; AD Instruments, Colorado Springs, CO). Every 15 min, hemodynamic and respiratory parameters were registered. A recruitment manoeuver (30 cm H_2O for 10 s) was performed at baseline, and then every 60 min, being the respiratory system compliance (C_{rs}) measured, by an end-inspiratory pause, before and after recruitment manoeuver.

Seven hours (420 min) after the start of MV, animals were euthanized by exsanguination.

Lung Injury Assessment

Pressure–Volume Curve Assessment. After 420 min of MV, a last recruitment maneuver was performed, and C_{rs} was measured before and after. Then, a pressure–volume curve was constructed by delivering three steps of 200 μl of inspiratory volume by the ventilator and measuring plateau airway pressure for each step. Three values of C_{rs} were obtained by calculating the ratio between the insufflated volume and the static pressure change. A mean value was then calculated.

Arterial Blood Gases Measurement. After 5 min of preoxygenation with F_{IO_2} of 1 (used for standardization of F_{IO_2} among animals), a blood sample was withdrawn from the catheter in the carotid artery and an aliquot (0.1 ml) analyzed with an I-STAT 1 portable analyzer (Burke & Burke, Milan, Italy).

Peripheral Total Leukocyte Count. Another aliquot from the same blood sample was used for the peripheral total leukocyte count. Twenty microliters of blood were suspended in 200 μl of Turk and leukocytes were counted with a Burker chamber.

Selective Bronchoalveolar Lavage. After animal exsanguination, the thorax was opened, and a macroscopic observation of the lungs was performed to identify the localization of the acid injury, seen as an hemorrhagic and nonrecruitable zone. Then, bronchoalveolar lavage (BAL) was performed separately for each lung, by excluding alternatively the right and left main bronchus. Lavage was performed three times for each lung, with 600 or 400 μl of lavage solution, respectively, for the right and left one. The lavage solution was composed of 0.9% saline solution and a protease inhibitor (Complete, Protease Inhibitor Cocktail Tablets; Roche Diagnostics GmbH, Mannheim, Germany). The BAL samples obtained were centrifuged for 10 min, 1,500 rpm, 4°C; the supernatant was conserved at −80°C for subsequent dosage of cytokines and albumin. Cell pellet was used for total leukocyte count (with a Burker chamber), and for differential cell count performed by Cytospin centrifugation (StatSpin Cytofuge 2; Bio-Optica, Milan, Italy), and stained with a modified Wright-Giemsa stain (Diff-Quick kit; Medion Diagnostics, Düdingen, Switzerland).

Myeloperoxidase Assay. Interstitial neutrophil infiltration was quantitated measuring myeloperoxidase activity, as previously described[22] (see also Supplemental Digital Content 2, http://links.lww.com/ALN/A943, which provides the description of methods and results referred to myeloperoxidase measurement).

Histological Evaluation. Two mice for $VILI_{25}$, $HCl\text{-}VILI_{15}$ and $HCl\text{-}VILI_{25}$ group, which did not undergo to BAL procedure were used for histological evaluation. Lungs were removed *en bloc* and fixed in 4% formalin for 24 h (at a pressure of 20 cm H_2O for the first 30 min) paraffin embedded and sectioned, as previously reported.[22] Three complete transverse hematoxylin and eosin stained sections (cranial, middle and caudal) of the right and left lung were viewed from each animal.

Albumin and Cytokines Assay. BAL fluid (BALf) supernatant was analyzed for albumin, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and macrophage inflammatory protein (MIP)-2 concentrations by enzyme-linked immuno sorbent assay, according to the manufacturer's instructions (Abnova Corporation, Taiwan; for Albumin and R&D systems, Minneapolis, MN for cytokines).

Cell counts, cytokines, albumin dosage, myeloperoxidase assessment, and histological examination were performed in a condition blinded to the assigned experimental group.

Baseline values for BAL cell count, leukocyte count, and cytokines measurement were obtained from five healthy mice.

Distribution of Ventilation by Computed Tomography

In a set of supplemental experiments we aimed at verifying the redistribution of V_T toward the left lung after the induction of right lung injury.

We studied three additional animals for each experimental group $HCl\text{-}VILI_{15}$, $HCl\text{-}VILI_{25}$, and $VILI_{25}$. These animals were ventilated according to the parameters used in each group, and underwent baseline computed tomography (CT) scan with a small animal micro-CT (Skyscan 1176, Bruker, Belgium), which was repeated 2 h after injury, at unmodified ventilatory settings.

The following acquisition parameters were used: 50 kV, 500 μA, 0.5 rotation step, 18-μm pixel size. Retrospective gating was used: by this approach several images are obtained during the respiratory cycle and airway pressure signal is used to sort, retrospectively, the images in three "bins", corresponding to inspiration, mid-exhalation, end-exhalation. Images were reconstructed with proprietary software of the scanner (NRecon, Skyscan). In blinded condition, left and right lung were manually outlined (CTan, Skyscan), on the inspiratory and end-expiratory images. The size of each lung during expiration and inspiration was computed as the product of the number of voxels by the voxel size (5.8×10^3 μm^3). The volume of gas was computed as the product of total volume by $CT_{mean}/(-1{,}000)$, where CT_{mean} is the average density, expressed in Hounsfield Unit of each lung; the Hounsfield Unit scale had been previously calibrated with a phantom of water and air, according to manufacturer suggestion. The fraction of V_T received by each lung was then calculated as the difference in gas volume between expiration and inspiration, normalized by the sum of the right and left lung.

Statistical Analysis

Data are expressed as mean ± SD. Comparisons of parametric variables involving all experimental groups were performed with one-way ANOVA, and when appropriate, Dunnett *post hoc* test was used to assess differences between each group and HCl-VILI$_{25}$. Kruskal–Wallis test, and Dunn multiple comparison test for *post hoc* were used for nonparametric measures (as cytokines, albumin concentrations). For compliance, peak inspiratory, and plateau pressure values of different time points we adopted two-way ANOVA. When appropriate, Bonferroni *post hoc* was used to compare other groups with HCl-VILI$_{25}$ at different time points. A regression analysis was performed to assess the correlation between hourly measured compliance of the respiratory system and time. Regional distribution of Vt obtained by micro-CT between VILI$_{25}$ and HCl-VILI$_{25}$ was analyzed by Mann–Whitney U test. *P* values of less than 0.05 were considered as statistically significant. Two-tailed statistics were used. Statistical analysis were performed by GraphPad PRISM 5.03 (GraphPad software Inc., San Diego, CA).

Results

We analyzed 14 mice for HCl-SB group; nine for HCl-VILI$_{15}$ group, 12 for HCl-VILI$_{25}$ group, and 11 for VILI$_{25}$. The sample size of groups reported in the results are for some variables different from these because some measures were missing due to technical problems in nonreproducible measurements (*e.g.,* impossible to withdraw arterial blood gas from the arterial carotid). These cases, however, were a few limited exceptions, evenly distributed among the different experimental groups.

Hemodynamic Parameters

We adjusted the fluid infusion rate to maintain hemodynamic stability (table 1). As a result, fluid input was 14 ± 2 ml·kg^{-1}·h^{-1} for HCl-VILI$_{15}$ group (*n 9*), 17 ± 4 ml·kg^{-1}·h^{-1} for HCl-VILI$_{25}$ (*n 12*), and 18 ± 4 ml·kg^{-1}·h^{-1} for VILI$_{25}$ (*n 11*; *P* = 0.03 in one-way ANOVA, no *post hoc* comparison resulted significant).

Gas Exchange

At the end of the experiment (420 min) $Pa_{O_2}/F_{I_{O_2}}$ was less than 300 mmHg and similar in all groups receiving HCl. Pa_{O_2} was significantly lower in HCl-VILI$_{25}$ compared with VILI$_{25}$ (*P* < 0.001 in one-way ANOVA). Pa_{CO_2} resulted similar in all groups (*P* = 0.06 in one-way ANOVA). All the arterial blood gas variables analyzed are reported in table 1. Sample size was: 14 HCl-SB mice; seven HCl-VILI$_{15}$ mice; nine HCl-VILI$_{25}$ mice; seven VILI$_{25}$ mice.

Respiratory Parameters

Mean peak inspiratory pressure and plateau airway pressure were significantly higher in HCl-VILI$_{25}$ *(n 12)* group compared with HCl-VILI$_{15}$ *(n 9)* and VILI$_{25}$ *(n 11*; *P* < 0.001 in one-way ANOVA). Peak inspiratory (Ppeak) and plateau pressure (Pplat) values at baseline, 180, and 420 min were compared between groups: for Ppeak, two-way ANOVA resulted significant (*P* < 0.001) for treatment but not significant (*P* = 0.052) for time and (*P* = 0.58) for interactions; for Pplat, treatment, time, and interactions resulted statistically significant (*P* < 0.001). *Post hoc* comparisons and values are reported in table 2.

Lung Mechanics

C_{rs} was measured by end-inspiratory pause in all ventilated groups at baseline and every hour, until the end of the experiment, before and after a RM (30 cm H_2O for 10 s). In figure 1A the temporal trend of C_{rs} is represented. From the first measurement performed after injury induction (60 min) until the end of experiment (420 min), HCl-VILI$_{25}$ showed a worse compliance compared with VILI$_{25}$ but not different compared with HCl-VILI$_{15}$ (two-way ANOVA resulted significant for time, treatment and interactions, *P* < 0.001; *post hoc* statistically significant tests are reported in fig. 1A). However, only in the HCl-VILI$_{25}$ group, we observed a worsening of mechanical properties, although mild, during the 7-h protocol, as shown

Table 1. Hemodynamic Parameters and Arterial Blood Gas Analysis

Group	Arterial Pressure, mmHg	Heart Rate, Breaths/Min	pH	Pa_{O_2}, mmHg	Pa_{CO_2}, mmHg	BE	HCO_3^-, mEq
HCl-VILI$_{15}$	102 ± 19	577 ± 61	7.27 ± 0.07	242 ± 111	36 ± 7	−10 ± 5	17 ± 4
HCl-VILI$_{25}$	105 ± 26	549 ± 48	7.19 ± 0.07	210 ± 54	48.1 ± 12	−9.7 ± 6	18 ± 5
VILI$_{25}$	106 ± 26	521 ± 76	7.27 ± 0.09	479 ± 83*	50 ± 12	−4 ± 6	26 ± 11*
HCl-SB	—	—	7.17 ± 0.09	277 ± 144	47 ± 12	−11.9 ± 4	17 ± 3

Hemodynamic parameters: mean ± SD of invasive arterial pressure and heart rate during the whole experiment for groups of mice that were mechanically ventilated (HCl-VILI$_{15}$; *n* 9 and HCl-VILI$_{25}$; *n* 12; VILI$_{25}$; *n* 11). Arterial blood gas analysis (mean ± SD) was performed at the end of protocol for 14 HCl-SB mice; seven HCl-VILI$_{15}$ mice; nine HCl-VILI$_{25}$ mice; seven VILI$_{25}$ mice: ANOVA resulted statistically significant for pH, and BE (*P* = 0.009 and *P* = 0.01) but no differences were seen for *post hoc* comparisons. Groups are: HCl-VILI$_{15}$ and HCl-VILI$_{25}$, which were instilled in the right bronchus with HCl and subjected to mechanical ventilation with tidal volume of 15 or 25 ml/kg for 7 h; HCl-SB that was instilled with HCl in right bronchus and left spontaneous breathing for 7 h; VILI$_{25}$ that was only ventilated with tidal volume of 25 ml/kg for 7 h.

* *P* < 0.05 *vs.* HCl-VILI$_{25}$.

BE = base excess; HCl = hydrochloric acid; Pa_{CO_2} = arterial carbon dioxide tension; VILI = ventilator-induced lung injury.

Table 2. Ventilatory Parameters

Group	BW, g	RR, breaths/min	V_T, µl	PEEP, cm H_2O	$Ppeak_{mean}$, cm H_2O	$Pplat_{mean}$, cm H_2O	Time, min	Ppeak, cm H_2O	Pplat, cm H_2O
HCl-VILI$_{15}$	24 ± 1	130	358 ± 18*	2 ± 0.2	17 ± 1*	12 ± 0.5*	0	15.8 ± 1.6*	10.4 ± 0.5*
							180	17.4 ± 0.6*	12.1 ± 0.7*
							420	17.3 ± 0.6*	12.5 ± 0.9*
HCl-VILI$_{25}$	24.7 ± 1.6	100	628 ± 31	2 ± 0.1	24.8 ± 1.8	19.3 ± 1.7	0	24.5 ± 3.2	16.5 ± 1.5
							180	25.5 ± 2.2	19.3 ± 2
							420	26.5 ± 3.1	21 ± 2.9
VILI$_{25}$	23.3 ± 1	100	616 ± 37	1.9 ± 0.2	23.3 ± 1*	15.5 ± 0.7*	0	23.6 ± 2	16.6 ± 1.3
							180	23.2 ± 1.1*	15.1 ± 0.9*
							420	24.2 ± 1.4*	15.7 ± 1.1*

Ventilatory parameters: mean ± SD. PEEP, Ppeak and Pplat were measured after recruitment manoeuvres (performed hourly). Represented in the table as mean of all registrations (7 h of experiment) and at different time points (baseline, 0 min; 180 and 420 min) of nine HCl-VILI$_{15}$; 12 HCl-VILI$_{25}$ and 11 VILI$_{25}$. Groups are: HCl-VILI$_{15}$ and HCl-VILI$_{25}$, which were instilled in the right bronchus with HCl and subjected to mechanical ventilation with tidal volume of 15 or 25 ml/kg for 7 h; VILI$_{25}$ that was only ventilated with tidal volume of 25 ml/kg for 7 h.

* $P < 0.05$ *vs.* HCl-VILI$_{25}$.

BW = body weight; HCl = hydrochloric acid; PEEP = positive end-expiratory pressure; Ppeak = peak inspiratory pressure; Pplat = plateau airway pressure; RR = respiratory rate; VILI = ventilator-induced lung injury; V_T = tidal volume.

by significant correlation between C_{rs} and time (R^2, 0.86, $P < 0.001$ for HCl-VILI$_{25}$; R^2, 0.16, $P = 0.4$ for HCl-VILI$_{15}$; R^2, 0.005, $P = 0.9$ for VILI$_{25}$); the value of C_{rs} before the instillation of acid was excluded from the correlation. At the end of experiment, pressure–volume curve was obtained from animals of each group (13 HCl-SB, nine HCl-VILI$_{15}$, 12 HCl-VILI$_{25}$, and 11 VILI$_{25}$): the mean C_{rs} was significantly lower in HCl-VILI$_{25}$ compared with VILI$_{25}$ (0.029 ± 0.0046 *vs.* 0.038 ± 0.0048 ml/cm H_2O, respectively, $P < 0.05$ for Dunnett test, and $P < 0.001$ in one-way ANOVA; fig. 1B).

Cellular Content in BAL

Figure 2 shows the total and differential cell counts in right (fig. 2A) and left (fig. 2B) lung. Interestingly, total cells and polymorphonuclear cells number were significantly higher in HCl-VILI$_{25}$ compared with other groups, both in the right (total cells: $P = 0.003$ in one-way ANOVA; polymorphonuclear cells: $P = 0.0037$ in one-way ANOVA) and in the left lung (total cells: $P = 0.0012$ in one-way ANOVA; polymorphonuclear cells: $P < 0.001$ in one-way ANOVA). Sample size was: 14 for HCl-SB group; eight for HCl-VILI$_{15}$, HCl-VILI$_{25}$, and VILI$_{25}$ group.

Peripheral Total Leukocyte Count

We observed a systemic propagation of local inflammatory process as demonstrated by leukocyte count. In HCl-VILI$_{25}$ group leukocyte count resulted significantly higher compared with the other groups ($P = 0.0052$ in one-way ANOVA, *post hoc* comparisons in fig. 3). Sample size was: 14 for HCl-SB; seven for HCl-VILI$_{15}$; nine for HCl-VILI$_{25}$; and seven for VILI$_{25}$ group.

Myeloperoxidase Activity

We show in Supplemental Digital Content 2 (http://links.lww.com/ALN/A943) that myeloperoxidase activity, an

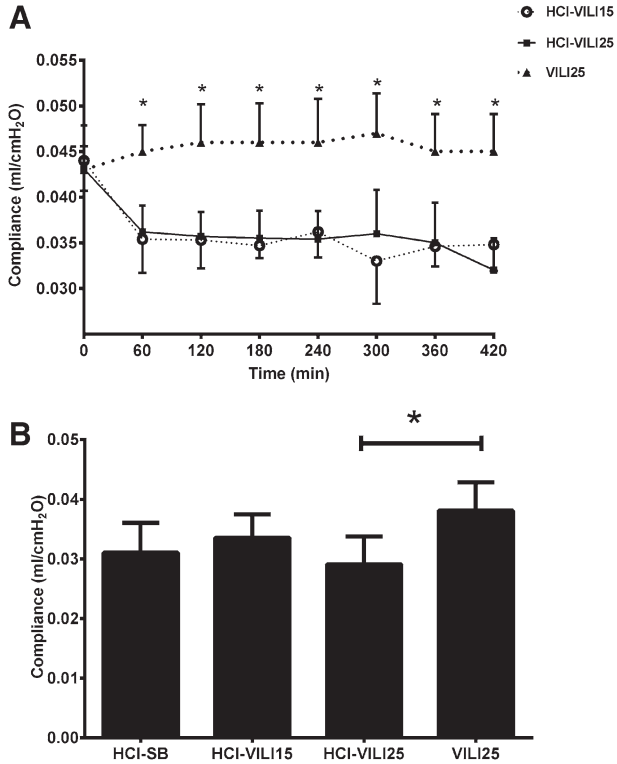

Fig. 1. (*A*; mean ± SD) Shows the temporal trend of respiratory system compliance (C_{rs}) of mechanically ventilated groups (HCl-VILI$_{15}$ *n* 9; HCl-VILI$_{25}$ *n* 12; VILI$_{25}$ *n* 11), and (*B*; mean ± SD) shows the mean C_{rs} from *pressure–volume curve* obtained at the end of experiment (HCl-SB *n* 13). Groups are: HCl-VILI$_{15}$ and HCl-VILI$_{25}$, which were instilled in the right bronchus with HCl and subjected to mechanical ventilation, with tidal volume of 15 or 25 ml/kg for 7 h; HCl-SB that was instilled with HCl in right bronchus and left spontaneous breathing for 7 h; VILI$_{25}$ that was only ventilated with tidal volume of 25 ml/kg for 7 h. **P* < 0.05 *versus* HCl-VILI$_{25}$ at the same time point. HCl = hydrochloric acid; VILI = ventilator-induced lung injury.

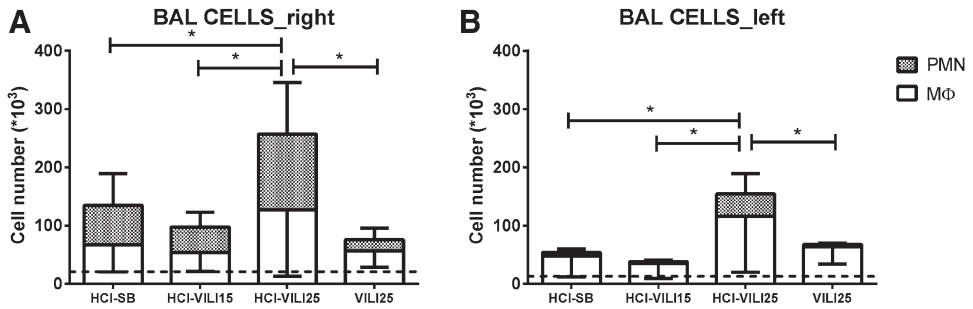

Fig. 2. Total and differential cell count in bronchoalveolar lavage from all groups for right (*A*) and left (*B*) lung at the end of experiment. *Dotted lines* represent baseline values from five healthy mice. Data are mean ± SD. Both total cell number and PMN number are significantly higher in HCl-VILI$_{25}$ (*n* 8) compared with the other groups (HCl-SB *n* 14; HCl-VILI$_{15}$ *n* 8; VILI$_{25}$ *n* 8). Groups are: HCl-VILI$_{15}$ and HCl-VILI$_{25}$, which were instilled in the right bronchus with HCl and subjected to mechanical ventilation, with tidal volume of 15 or 25 ml/kg for 7 h; HCl-SB that was instilled with HCl in right bronchus and left spontaneous breathing for 7 h; VILI$_{25}$ that was only ventilated with tidal volume of 25 ml/kg for 7 h. *P < 0.05. BAL = bronchoalveolar lavage; HCl = hydrochloric acid; Mϕ = macrophages; PMN = polymorphonuclear cells; VILI = ventilator-induced lung injury.

indirect indicator of neutrophil infiltration of interstitial lung areas, resulted in an increase in both right and left lungs of all experimental groups, and in particular, in HCl-VILI$_{15}$ right lung.

Histologic Findings

In figure 4 we reported representative histological images from lungs of VILI$_{25}$, HCl-VILI$_{15}$, and HCl-VILI$_{25}$ group. We did not perform histological analysis by using injury scoring, but we qualitatively evaluated sections of lung apex-medium and basal lung level. A low or moderate degree of injury was observed in VILI$_{25}$ lungs, it was mainly characterized by presence of proteinaceous edema, a few hyaline membranes, inflammatory cells (macrophages), and some erythrocytes in alveolar spaces. Lungs of HCl-treated mice exhibited conspicuous alveolar and perivascular proteinaceous edema and alveolar hemorrhage; hyaline membranes are the typical features of HCl-treated lungs. Leukocytes infiltrates (macrophages and polymorphonuclear cells), alveolar septal thickening, and alveolar hemorrhage were more abundant HCl-VILI$_{25}$ lungs than in those of HCl-VILI$_{15}$ group. Right lungs (that underwent HCl instillation) showed more pronounced structural changes and inflammatory process compared with their contralateral lungs and compared with lungs of VILI$_{25}$ group.

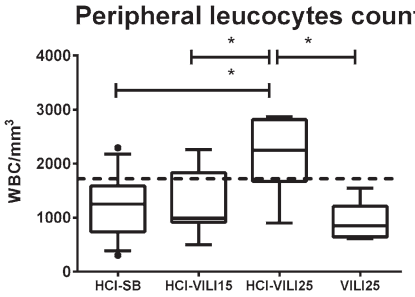

Fig. 3. Leukocyte count at the end of experiment in all study groups (HCl-SB; *n* 14; HCl-VILI$_{15}$; *n* 7 and HCl-VILI$_{25}$; *n* 9; VILI$_{25}$; *n* 7). *Dotted lines* represent baseline values from five healthy mice. Data are expressed as *box* (median, 25th and 75th percentiles) and *whiskers* (10th and 90th percentiles). Groups are: HCl-VILI$_{15}$ and HCl-VILI$_{25}$, which were instilled in the right bronchus with HCl and subjected to mechanical ventilation, with tidal volume of 15 or 25 ml/kg for 7 h; HCl-SB that was instilled with HCl in right bronchus and left spontaneous breathing for 7 h; VILI$_{25}$ that was only ventilated with tidal volume of 25 ml/kg for 7 h. *P < 0.05. HCl = hydrochloric acid; VILI = ventilator-induced lung injury; WBC = white blood cell.

Albumin and Cytokines Assay in BALf

Figure 5 shows albumin concentration in BALf. HCl-treated group exhibited a similar increase in the values of albumin both in the right, and in the left lung. Albumin concentration resulted significantly higher in HCl-VILI$_{25}$ group as compared with VILI$_{25}$ both in the right, and in the left lung. Concentration of cytokines analyzed in BALf (TNFα; IL-1β; MIP-2) did not show statistically significant differences between groups, either for the right, or the left lung (for right lung: IL-1β: P = 0.16; for left lung: TNFα: P = 0.49; IL-1β: P = 0.5; MIP-2: P = 0.28 in the Kruskal–Wallis test), except for TNFα and MIP-2 in the right lung. In those cases, the Kruskal–Wallis test revealed a statistical significance (P = 0.016 and P = 0.014, respectively), but no significant differences were disclosed by the *post hoc* analysis (fig. 5). Sample size was of 6–10 for the group for each protein.

Tidal Volume Distribution

Figure 6 shows the relative distribution of V_T in the right and left lung in three animals of HCl-VILI$_{15}$, HCl-VILI$_{25}$, and VILI$_{25}$ groups at baseline (fig. 6A) and after the induction of injury (fig. 6B): it can be noticed that the unilateral lung injury promoted a redistribution of V_T toward the left lung, similar in HCl-VILI$_{15}$ and HCl-VILI$_{25}$, whereas no redistribution is seen in the VILI$_{25}$ group. In particular, the fraction of V_T distributed to the left lung was greater in the HCl-VILI$_{25}$, compared with the VILI$_{25}$ (P = 0.05) after injury of the opposite lung, and no difference was seen at baseline.

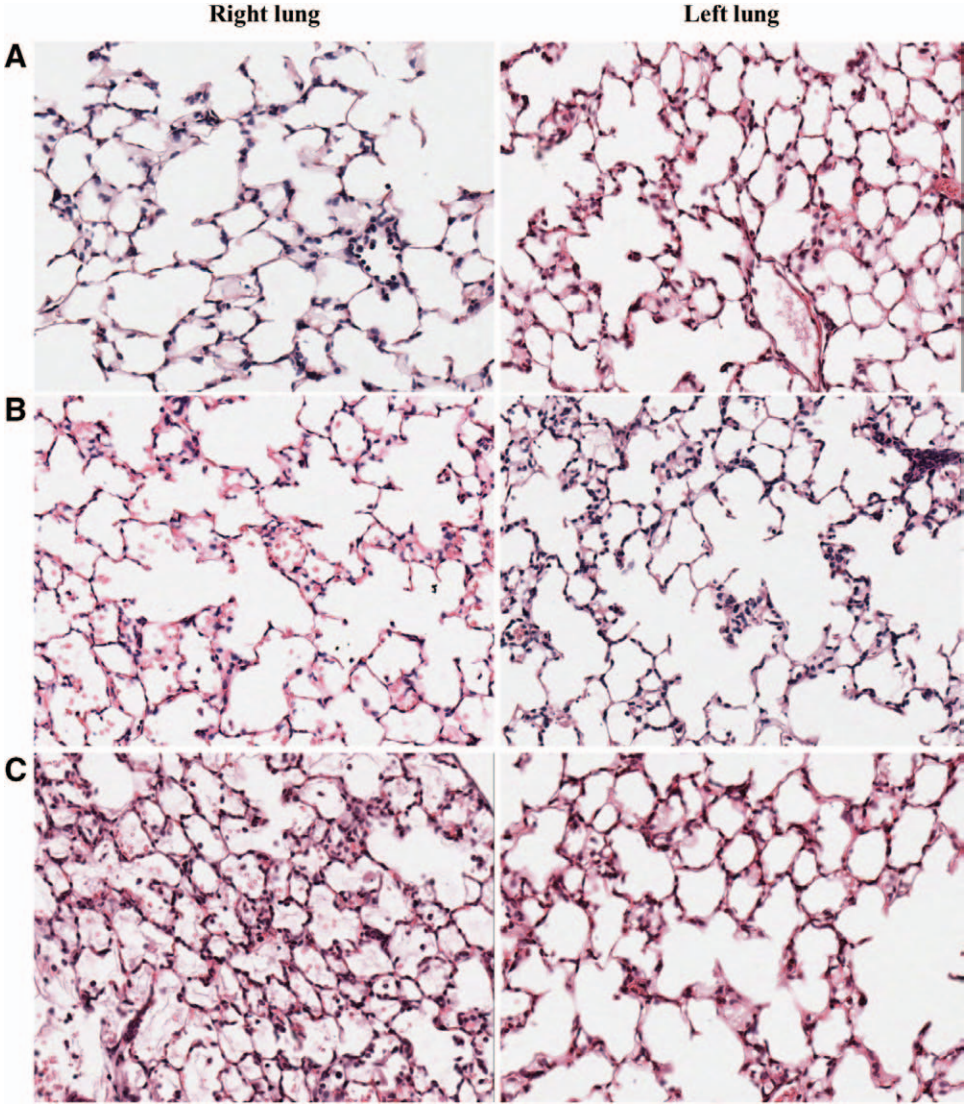

Fig. 4. Typical images of histological sections of right and left lung of $VILI_{25}$ (*A*); $HCl\text{-}VILI_{15}$ (*B*); and $HCl\text{-}VILI_{25}$ (*C*) groups. Hematoxylin and eosin, ×400 magnification. Groups are: $HCl\text{-}VILI_{15}$ and $HCl\text{-}VILI_{25}$, which were instilled in the right bronchus with HCl and subjected to mechanical ventilation, with tidal volume of 15 or 25 ml/kg for 7 h; $VILI_{25}$ that was only ventilated with tidal volume of 25 ml/kg for 7 h. HCl = hydrochloric acid; VILI = ventilator-induced lung injury.

Discussion

In this study, we described an *in vivo* model of "two-hit" lung injury caused by unilateral acid aspiration pneumonitis, with the superimposition of a prolonged nonprotective MV. As many authors suggested, a preinjured lung is more susceptible to MV, particularly, if nonprotective V_T (more than 6–8 ml/kg) are adopted.[2–4,23] We observed that injurious MV not only aggravated the preexisting injury (in the right lung), but also harmed the contralateral lung, which was not directly damaged otherwise.

We decided to deliver a V_T lower than those reported by other groups,[12,24–26] with a lower hemodynamic impact, to allow the *in vivo* experiment to last more than 2–4 h. However, we planned to prolong the ventilation for a relatively long time span, potentially adequate to evaluate the effect of therapeutic strategies because this is one of the goals of the model described. In the present work, we compared the effects of prolonged MV with V_T of 25 *versus* 15 ml/kg or *versus* spontaneous breathing on the previously injured lung. We observed that MV with high V_T is associated with both a worsening of the preexisting damage, and an increased inflammation and permeability in the not directly injured lung, likely because of a redistribution of regional V_T toward this lung.

We focused on both functional and inflammatory features. The high V_T group (25 ml/kg) showed an alteration of gas exchange mainly attributable to acid aspiration because we did not find any difference in the level of arterial hypoxemia among all groups receiving HCl instillation. Conversely the ventilation with a V_T of 25 ml/kg, induced a progressive deterioration of the respiratory system

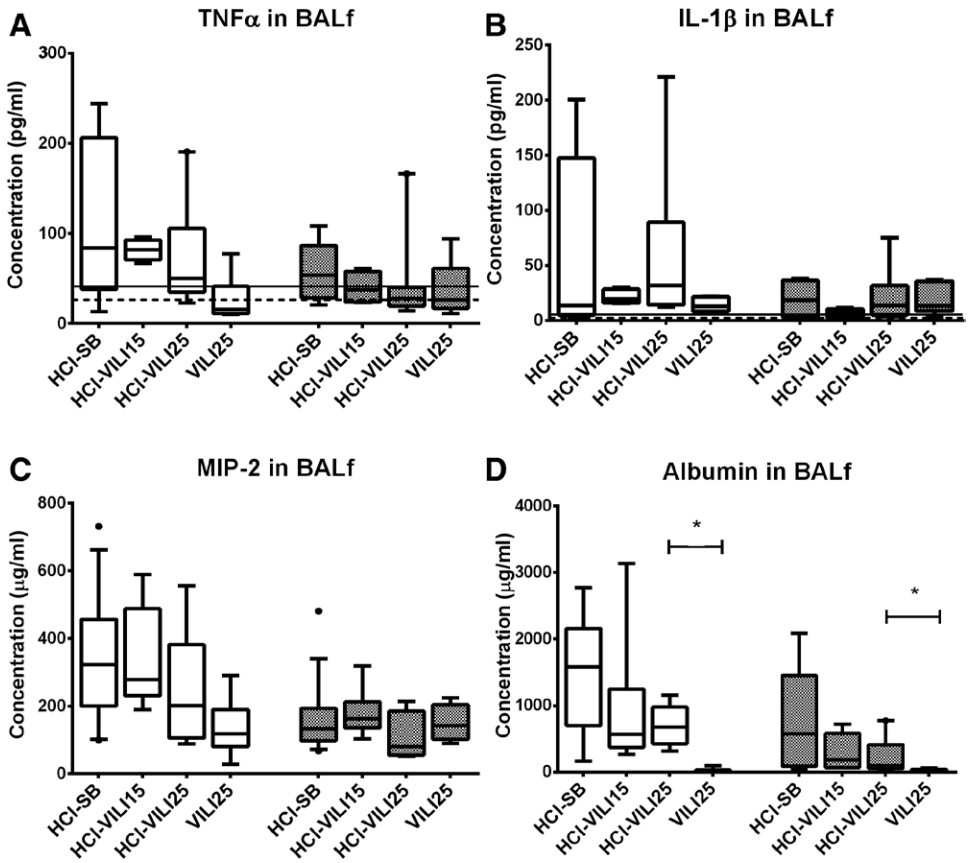

Fig. 5. Albumin and cytokines concentration in free-cell bronchoalveolar lavage fluid (BALf) at the end of experiment. For cytokines, baseline values from five healthy mice were indicated in graphs: *continuous line* for right lung and *dotted line* for left lung. In those mice, macrophages inflammatory protein (MIP)-2 resulted under the detection limit. Data are expressed as *box* (median, 25th and 75th percentiles) and *whiskers* (10th and 90th percentiles). *N* 6–10/group for each protein. *White columns* represent the right lung and *grey columns* the left lung. For albumin (*D*), HCl-VILI$_{25}$ had an increased content compared with VILI$_{25}$ group in both lungs. *$P < 0.05$. Tumor necrosis factor (TNF)-α (*A*) and MIP-2 (*C*) levels in the right lung one-way ANOVA resulted significantly different ($P = 0.016$ and $P = 0.014$, respectively), although no difference was revealed by the *post hoc* comparisons. No differences were seen for interleukin (IL)-1β (*B*). Groups are: HCl-VILI$_{15}$ and HCl-VILI$_{25}$, which were instilled in the right bronchus with HCl and subjected to mechanical ventilation, with tidal volume of 15 or 25 ml/kg for 7 h; HCl-SB that was instilled with HCl in right bronchus and left spontaneous breathing for 7 h; VILI$_{25}$ that was only ventilated with tidal volume of 25 ml/kg for 7 h. HCl = hydrochloric acid; VILI = ventilator-induced lung injury. In *C, dots* indicate outliers.

compliance, when delivered on acid-injured lungs. This worsening was probably caused by the injurious ventilation because a decline in respiratory system compliance was observed only in the time course of the animals ventilated with V_T of 25 ml/kg, but not in those ventilated with 15 ml/kg. When we evaluated the effects of MV on inflammatory cells and alveolar–endothelial barrier alterations (assessed as albumin concentration in BALf), we showed that, as expected, the number of neutrophils (main effectors of VILI)[27] and endothelial permeability were markedly increased in the right lung exposed to a two-hit injury, using a higher V_T (25 ml/kg). However, the left lung also, despite no direct injury by acid instillation, did show an increased alveolar permeability and cellular infiltrate when ventilated with V_T 25 ml/kg. Interestingly, MV *per se*, even with a V_T of 25 ml/kg was unable to cause severe injury, unless in the presence of a previous injury delivered to the right lung. One potential explanation for this finding is that because injury in the right lung induces a loss of aeration, as demonstrated from previous studies,[17–19,21] the injury in the ventilated lung seems to be related to the ratio between V_T and lung volume available for ventilation; indeed, as shown by our additional experiments reported in figure 6, the induction of unilateral injury in the right lung determined a shift of ventilation toward the left one. Other potential mechanisms could underlie the presence of an inflammatory response in the left lung, in particular, the HCl challenge brought might lead to secretion of inflammatory mediators to the right lung, causing neutrophil sequestration in the contralateral also, as shown previously[28,29] by our group.[22] This might explain the similar levels of albumin seen in the BALfs in the left lung of all HCl-treated animals. However, injury was further increased by VILI, as shown by the higher number of leukocytes and neutrophils measured in the left

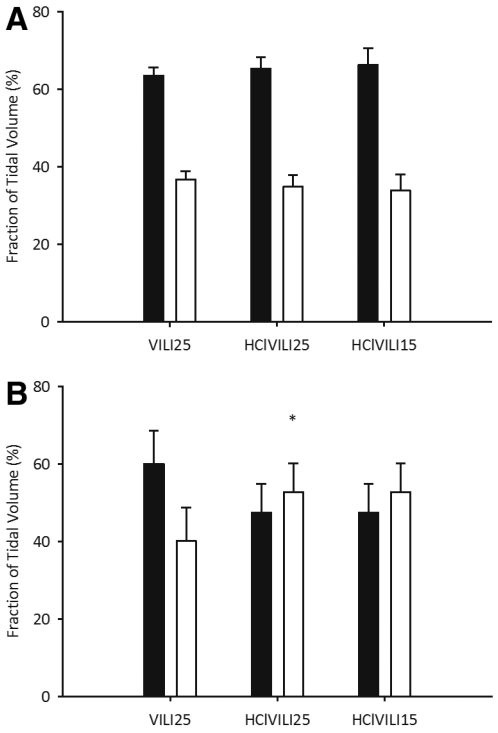

Fig. 6. Relative distribution of tidal volume in the right (*black bars*) and left lung (*white bars*) in animals of the HCl-VILI$_{15}$, HCl-VILI$_{25}$ and VILI$_{25}$ groups (n = 3 for each group) at baseline (*A*) and after the induction of injury (*B*): it can be noticed that the unilateral lung injury promotes a redistribution of tidal volume toward the left lung, whereas no redistribution is seen in the VILI$_{25}$ group. Groups are: HCl-VILI$_{15}$ and HCl-VILI$_{25}$, which were instilled in the right bronchus with HCl and subjected to mechanical ventilation, with tidal volume of 15 or 25 ml/kg for 7 h; VILI$_{25}$ that was only ventilated with tidal volume of 25 ml/kg for 7 h. * P = 0.05 *versus* omolateral lung of VILI$_{25}$ group (Mann–Whitney U test). HCl = hydrochloric acid; VILI = ventilator-induced lung injury.

BALf and by the worst histological findings in animals of the HCl-VILI$_{25}$ group as compared to HCl-VILI$_{15}$. The group undergoing spontaneous breathing is more difficult to interpret: first a high spontaneous V_T generated by animals can be as injurious as a mechanically delivered one[30]; second the absence of positive end-expiratory pressure could have allowed further alveolar derecruitment in both lungs, with the subsequent development of inflammation.[31]

It is known that VILI is mediated by proinflammatory cytokines and chemokines, including TNFα, MIP-2, and IL-6. Studies showed that VILI induces overexpression of these cytokines, detectable in BALf, in lung homogenate, or systemically. In the present study, we did not find any solid association between the cytokines concentration in BALf and V_T. The direct pulmonary insult triggered by acid instillation was associated with an increased intraalveolar level of these inflammatory mediators, but MV with high V_T did not have an additional effect on cytokines concentration. In keeping with the findings by Wilson *et al.*[12], TNFα was similar in all groups, probably because it has a transient elevation in the early phases (120 min) of MV but not in the later phases (more than 180 min). We also did not detect any difference in IL-1β and MIP-2 concentration among groups. These results suggest that TNFα, MIP-2, and IL-1β in BALf might be not suitable for evaluating the effect of therapeutic strategies in this specific two-hit model after 7 h of MV.

Our study has several limitations: first, in our experimental protocol V_T was set according to body weight and not to inspiratory capacity. Even if our method was used in most of the published studies, it might be associated to a greater variability of lung injury severity. Second, although only few studies investigated *in vivo* model of MV lasting more than 6 h, we evaluated the effects of MV (inflammatory process and gas exchange) only after 7 h, so we cannot speculate on the potential evolution of lung injury. Third, the puzzling results in our study regarding the absence of a signal in the cytokines concentration might suggest that lung homogenate might probably be more accurate than BALf.[4] Finally we did not measure the circulating levels of lactate; this might have explained the reason of metabolic acidosis in HCl-treated groups because one possible reason would be an impaired peripheral perfusion, despite normal blood pressure.

In conclusion, in this study, we described a two-hit model of acute lung injury, in which one lung received both acid instillation and injurious ventilation. The contralateral lung is injured by the effect of MV only, probably as a consequence of the reduced volume accessible to ventilation. Due to these characteristics, we believe that this model bears elements of resemblance with the VILI occurring in the clinical scenario and that for this reason, it might be useful in evaluating the effect of new therapeutic strategies.

The authors thank Alice Grassi, M.D., Resident, Department of Health Science, University of Milan-Bicocca, Monza (MB), Italy, for her support in the *in vivo* experiments.

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