

Neutrophil Stimulation with Granulocyte Colony-stimulating Factor Worsens Ventilator-induced Lung Injury and Mortality in Rats

Waheedullah Karzai, M.D.,* Xizhong Cui, M.D., Ph.D.,† Norbert Heinicke, M.D.,‡ Christian Niemann, M.D.,§ Eric P. Gerstenberger, || Rosaly Correa, M.D.,# Steven Banks, Ph.D.,|| Bjoern Mehlhorn, M.D.,** Frank Bloos, M.D., Ph.D.,†† Konrad Reinhart, M.D.,‡‡ Peter Q. Eichacker, M.D.§§

Background: Based on the association between the neutrophil and ventilator-induced lung injury, the authors hypothesized that neutrophil inhibition with fucoidin would be beneficial and stimulation with granulocyte colony-stimulating factor (G-CSF) would be harmful in a rat model of lethal ventilator-induced lung injury.

Methods: Animals (n = 111) were randomly assigned to be pretreated with fucoidin, G-CSF, or placebo (control) before 4 h of low-tidal-volume (10 ml/kg) or high-tidal-volume (40 ml/kg) mechanical ventilation.

Results: All low-volume animals survived. With high volumes, compared with controls, fucoidin did not improve survival (3 of 20 control animals and 5 of 20 fucoidin animals died; $P = 0.51$) but G-CSF significantly worsened it (18 of 22 animals died; $P < 0.001$). Circulating neutrophils were increased early with G-CSF and late with fucoidin with low and high tidal volumes ($P < 0.05$ for each treatment and tidal volume). Fucoidin decreased lung neutrophils, but these were only significant with high tidal volumes, whereas G-CSF increased lung neutrophils but only significantly with low tidal volumes ($P \leq 0.01$ for each). Fucoidin did not alter any cardiopulmonary measure significantly. Compared with control, G-CSF increased airway pressures with high tidal volumes and worsened lung edema and arterial oxygen with both tidal volumes ($P < 0.05$ for each).

Conclusions: In this model, neutrophil stimulation by G-CSF increased lung dysfunction and with high tidal volumes worsened survival rates. Extrapolated clinically, neutrophil stimulation either by agents such as G-CSF or conditions such as sepsis may aggravate ventilator-induced lung injury.

PATIENTS with acute lung injury requiring mechanical ventilation due to infection, aspiration, or other causes are also at risk of ventilator-induced lung injury (VILI).^{1,2} The pathogenesis of this injury relates in part to overdistension and/or cyclic collapse of distal airways and alveoli. Although a primary goal with mechanical ventilation is to minimize tidal volumes and airway pressures to limit such injury, if the acute lung injury itself is severe, overdistension of alveolar spaces may still occur

in remaining areas of lung in which normal compliance has been preserved.³

The pathogenesis of VILI may relate in part to the activation and release of host inflammatory mediators after mechanical injury to alveolar epithelial and endothelial cells.^{4,5} The neutrophil has been implicated in this inflammatory injury.^{4,5} Mechanical ventilation for prolonged periods in normal animals is associated with pulmonary neutrophil recruitment.⁶⁻⁹ Neutrophils are prominent in bronchoalveolar lavage fluid from patients with acute lung injury and evidence of pressure- or volume-related injury.¹⁰ Normal neutrophils can be activated when exposed to bronchoalveolar lavage fluid from patients with acute lung injury receiving mechanical ventilation *via* conventional *versus* protective strategies.^{11,12} Finally, it has been suggested in some animal models that neutrophil inhibition or depletion reduces VILI.^{13,14}

To further define the role of the neutrophil in lung injury related to mechanical ventilation alone, we compared the effects of inhibiting or stimulating neutrophil function in normal uninjured rats mechanically ventilated for 4 h with low (10 ml/kg) or high (40 ml/kg) tidal volumes. We hypothesized that neutrophil inhibition would be beneficial and stimulation would be harmful in normal rats ventilated with high but not low tidal volumes. To inhibit neutrophil function, we pretreated animals with fucoidin, a sulfated fucose polymer, which interferes with selectin-mediated neutrophil adhesion to endothelial cells.¹⁵ Fucoidin has been shown to limit neutrophil recruitment and tissue injury in the lung and other organs in rats and rabbits.^{16,17} To stimulate neutrophil function, we pretreated animals with recombinant granulocyte colony-stimulating factor (G-CSF). Recombinant G-CSF simulates the actions of endogenous G-CSF and is a potent stimulator of circulating neutrophil number and function.¹⁸ The regimen of G-CSF we used has been shown to increase circulating and lung neutrophil number in rats challenged with both noninfectious (*i.e.*, hyperoxic inspired gas mixtures) and infectious (*i.e.*, gram-negative bacteria) challenges.¹⁹ We measured systemic hemodynamics and pulmonary function, circulating and lung lavage neutrophils, lung histology, and lung lavage protein concentrations. Because activation and release of tumor necrosis factor (TNF)- α and interleukin (IL)-6 have been associated in clinical studies with the inflammatory injury occurring during VILI, we also

* Reader in Anesthesiology and Head, Department of Anesthesiology, ‡ Resident, Department of Internal Medicine, § Resident, Department of Anesthesiology, ** Staff Anesthesiologist, †† Attending Anesthesiologist, ‡‡ Professor of Anesthesiology and Critical Care Medicine, Department of Anesthesiology and Critical Care Medicine, Friedrich-Schiller-University Hospital. † Research Scientist, || Statistician, # Pathologist, §§ Senior Investigator, Critical Care Department, National Institutes of Health.

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Address reprint requests to Dr. Karzai: Department of Anesthesia and Intensive Care, Zentralklinik Bad Berka, Robert-Koch-Allee 9, 99437 Bad Berka, Germany. Address electronic mail to: ana@zentralklinik-bad-berka.de. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

measured these two cytokines in blood and lung lavage.¹⁰

Materials and Methods

Animal Care and Use

The experimental protocol for this study was approved by the local Animal Protection Committee (Regierungspräsidium Weimar, Germany). Animals were kept in groups of three or four and were allowed food and water before beginning the study.

Study Design

After treatment with G-CSF, fucoidin, or placebo (control), anesthetized and paralyzed male Wistar rats weighing between 350 and 400 g were mechanically ventilated for 4 h with either low (10 ml/kg) or high (40 ml/kg) tidal volumes. Immediately before initiation of mechanical ventilation, animals had central venous, carotid arterial, and tracheostomy catheters placed. The following measures were obtained during mechanical ventilation: systolic arterial pressure, heart rate, arterial blood gases, and mean airway pressures at baseline and 30-min intervals; complete blood counts at the beginning (0 h) and immediately before the completion of mechanical ventilation (4 h); and serum IL-6 and TNF concentrations at 4 h only. After measures at 4 h, animals were killed, and single lung lavage was performed for cell, protein, IL-6, and TNF determinations while the other lung was removed for histologic assessment of lung edema and alveolar neutrophils. Animals that died during the 4-h period had blood removed for complete blood counts, and lung lavage and histology were performed immediately at the time of death.

Animal Randomization and Study Group Sizes

In initial experiments, assignment cards blindly selected for each experiment were used to randomly assign animals to a combination of treatment (placebo, fucoidin, or G-CSF) and tidal volume (10 or 40 ml/kg). Placebo, fucoidin, and G-CSF, respectively, were administered to 19, 9, and 21 animals with low tidal volumes and 20, 20, and 22 with high tidal volumes. Overall, fewer animals were assigned to treatment with fucoidin and low tidal volumes because of limited availability of the study agent.

G-CSF, Fucoidin, and Antibiotic Treatments

Both G-CSF and fucoidin were diluted with phosphate-buffered saline (PBS). Animals were given G-CSF (100 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ subcutaneously; Amgen, Munich, Germany) 48 and 24 h before mechanical ventilation; fucoidin (4 mg followed by 6 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ intraperitoneally; Sigma, Deisenhofen, Germany) started 2 h before and continued until the completion of mechanical ven-

tilation; or, as placebo (control), PBS. Doses of G-CSF and fucoidin chosen for study were based on previously published studies and pilot experiments in rats.^{16,19} Control animals received PBS subcutaneously and intraperitoneally at time points simulating the scheduled administration of G-CSF and fucoidin, respectively. Animals receiving G-CSF were treated with intraperitoneal PBS at time points to simulate the scheduled fucoidin administration, whereas animals receiving fucoidin were treated with subcutaneous PBS to simulate the scheduled G-CSF administration. Therefore, all animals received equivalent volumes of drug or diluent based on a similar schedule. Although an aseptic technique was used for all procedures, to further prevent early infection related to skin or airway contamination, all animals received a single dose of cefuroxime (12.5 mg intramuscularly) immediately before mechanical ventilation.

Catheter and Tracheotomy Placement

Animals were initially anesthetized with intraperitoneal ketamine (20 mg/kg; Ayarost GmbH and Co., Twistringen, Germany) and midazolam (4 mg/kg; Hoffman-LaRoche AG, Grenzach-Whylen, Germany). Their necks were shaved and disinfected, and a 2-cm skin incision was made parallel to the trachea. The right external jugular and carotid artery were cannulated with plastic catheters (Primed Medizintechnik GmbH, Halberstedt, Germany). The trachea was exposed and cannulated with a blunt metal 16-gauge needle positioned with its tip above the tracheal bifurcation. The needle was secured, and the neck incision was sutured closed. Ventilation was started to ensure that no air leaks were present.

Anesthesia and Paralysis during Mechanical Ventilation

After catheter placement and initiation of mechanical ventilation, animals received intravenous loading doses followed by continuous infusions of ketamine (50 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) and midazolam (0.5 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$). Pancuronium was administered hourly (0.1 $\text{mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$; Curamed Pharma GmbH, Karlsruhe, Germany).

Ventilation Strategy

Using a volume-controlled small-animal ventilator (Technical & Scientific Equipments GmbH, Bad Homburg, Germany), rats were ventilated for 4 h with either low (10 ml/kg) or high (40 ml/kg) tidal volumes, all at a rate of 40 breaths/min, with a positive end expiratory pressure of 5 cm H₂O. These tidal volumes were chosen based on pilot studies showing that for the 4-h period of mechanical ventilation to be investigated, the low one, which generates peak inspiratory pressures of 9–10 mmHg (12–14 cm H₂O), was well tolerated, whereas the high one, which produces peak inspiratory pressures of 29–31 mmHg (41–43 cm H₂O), was associated with

lethality. For the purpose of changing only one variable related to minute ventilation, tidal volumes but not respiratory rates were altered between study groups. However, to achieve comparable partial alveolar and arterial oxygen and carbon dioxide pressures at baseline, animals receiving low tidal volume were ventilated with a fractional inspired oxygen concentration of 0.37 and a fractional inspired carbon dioxide concentration of 0, whereas animals receiving high tidal volume were ventilated with a fractional inspired oxygen concentration of 0.30 and an fractional inspired carbon dioxide concentration of 0.05 to produce an arterial carbon dioxide concentration of 40 mmHg.

Cardiopulmonary and Laboratory Analysis

After placement, catheters were connected to pressure transducers for arterial pressure and heart rate determinations (SMU 611; Hellige, Freiburg, Germany). Mean airway pressures were transduced and measured (SMU 611). Arterial blood was removed for blood gas determination (ABL 50; Radiometer, Copenhagen, Denmark), and venous blood was removed for complete blood counts and differentials and serum TNF- α and IL-6 determinations. Lung lavage fluid was centrifuged, and the cell pellet was resuspended in PBS. The number of cells in PBS was counted with a hemocytometer, and a differential count was determined on a smear using Wright stain. Lung lavage protein concentrations (Clinical System LX20; Beckman Synchron, Krefeld, Germany), and cytokine concentrations were determined on the supernatant. For histologic assessment of lung edema and neutrophils, an upper lobe bronchus was cannulated, and the lobe was infused and fixed with formaldehyde (4%) (Mallinkrodt Specialty Chemicals, Paris, KY), dehydrated, embedded in paraffin, and sectioned at 6 μ m. Lung tissue sections were then analyzed by an observer blinded to the study groups as previously described.¹⁹ Lung edema was scored on a scale of 0 (no edema evident) to 4 (maximum edema). Edema was characterized primarily by the presence of dilated alveolar spaces filled with amorphous and lightly acidophilic material (pink fluid), congestion of the alveolar walls, and dissociation of perivascular tissue generally associated with hemorrhagic foci. Alveolar neutrophils were enumerated by randomly examining 10 fields/slide at a magnification of $\times 480$ (oil immersion). Fields with large vessels were not evaluated. To measure rat serum and lung lavage TNF- α , we incubated 96-well plates with a 0.5% solution of rabbit anti-rat TNF- α serum. After 24 h of incubation at 4°C and washing, standard or test sera or lavage fluid was added, and the plates were incubated for 4 h at room temperature. After washing, a 1- μ g/ml concentrated biotin-conjugated anti- μ rat antibody (Pharmingen, San Diego, CA) was added. After repeated washing, 0.5 μ g/ml concentrated streptavidin-peroxidase conjugate solution (Dianova, Hamburg, Germany)

was added. After an additional 30 min, the wells were washed, and the peroxidase substrate 3,3',5,5'-tetramethylbenzidine (Sigma, Deisenhofen, Germany) was added. The reaction was stopped after 10–15 min with 1 M H₂SO₄, and the absorption of the solution was read at 450 nm. Serum and lavage IL-6 concentrations were performed using a commercial assay (Biosource, Camarillo, CA).

Statistical Methods

For the high-ventilation group, survival was analyzed using a Wilcoxon model with two one-degree-of-freedom tests (fucoidin *vs.* control and G-CSF *vs.* control). All other laboratory parameters were analyzed with an analysis of variance (ANOVA) taking into account time of measurement, level of mechanical ventilation, and treatment. Where either the drug main effect or interaction terms involving drug were significant, a Tukey test was performed. For mean airway pressure and arterial oxygen and carbon dioxide pressures, the effect of treatment did not differ over the 4 h of study ($P =$ not significant [NS] for all ANOVA), so the mean value across time for each animal was analyzed.²⁰ Measures from all animals were included in analysis up until the time of their death. Numbers of animals used for individual measures are shown on figures or in tables. Changes in oxygenation with high tidal volumes were also analyzed over the first 2 h of mechanical ventilation, when most animals were still present for analysis. A P value 0.05 or less was considered significant. In figures, P values are only shown for changes from control that reached significance.

Results

Comparison of G-CSF or Fucoidin with Placebo during 4 h of Low- or High-tidal-volume Ventilation

Survival Rates. All animals survived during mechanical ventilation with low tidal volumes (10 ml/kg) (19 control, 9 fucoidin, and 21 G-CSF animals). In contrast, mechanical ventilation with high tidal volumes (40 ml/kg) caused lethality in each of the three treatment groups (fig. 1). In controls with high tidal volumes, there were 3 nonsurvivors and 17 survivors ($P = 0.09$ compared with controls receiving low tidal volumes). Compared with these high-tidal-volume controls, fucoidin did not alter survival significantly (5 nonsurvivors and 15 survivors; $P = 0.51$ compared with controls, Wilcoxon), but G-CSF significantly worsened it (18 nonsurvivors and 4 survivors; $P = 0.001$ compared with controls, Wilcoxon).

Circulating Neutrophils. In controls, circulating neutrophils at the beginning and end of mechanical ventilation did not differ comparing low and high tidal volumes ($P =$ NS, ANOVA; fig. 2). Compared with con-

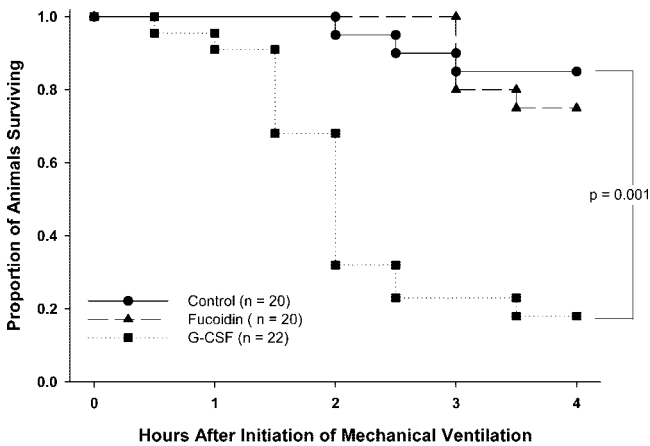


Fig. 1. Proportion of animals surviving *versus* time after initiation of high-tidal-volume (40 ml/kg) mechanical ventilation in controls or animals treated with fucoidin or granulocyte colony-stimulating factor (G-CSF). Compared with controls, fucoidin did not alter survival significantly, but G-CSF significantly reduced it.

trols, fucoidin with both low and high tidal volumes increased circulating neutrophils significantly at the end ($P = 0.002$ and $P < 0.0001$, respectively, ANOVA) but not the beginning ($P = \text{NS}$ for both, ANOVA) of ventilation. In contrast, compared with controls, G-CSF with both low and high tidal volumes increased circulating neutrophils significantly at the beginning ($P < 0.0001$ for both, ANOVA) but not the end ($P = \text{NS}$ for both) of ventilation.

Bronchoalveolar Lavage and Histologic Lung Neutrophils. At the end of mechanical ventilation in controls, lung lavage and histology neutrophils were increased with high compared with low tidal volumes ($P = 0.007$ and $P = 0.01$ for lavage and histology neutrophils, respectively, ANOVA; figs. 3A and B). Compared with controls, fucoidin decreased lung lavage and histology neutrophils with both low and high tidal volumes, but these differences were only significant for lavage neutrophils with high tidal volumes ($P = 0.003$, ANOVA). In contrast, compared with controls, G-CSF increased lung lavage and histology neutrophils with both tidal volumes, but these differences were only significant at low tidal volumes ($P = 0.004$ and $P = 0.01$ for lung lavage and histology neutrophils, respectively, ANOVA).

Cardiopulmonary Parameters. In all treatment groups (control, fucoidin, and G-CSF) from 0 to 4 h, systolic arterial blood pressure (data not shown) and heart rate (data not shown) with low and high tidal volumes and arterial oxygen and mean airway pressures with high tidal volumes decreased ($P \leq 0.05$ for all for the effect of time on changes in these parameters, ANOVA; table 1). During this time period in all treatment groups, compared with low tidal volumes, high ones were associated with increased mean airway pressures ($P = 0.0001$) and decreased arterial oxygen ($P = 0.04$), arterial carbon dioxide ($P = 0.002$), systolic arterial

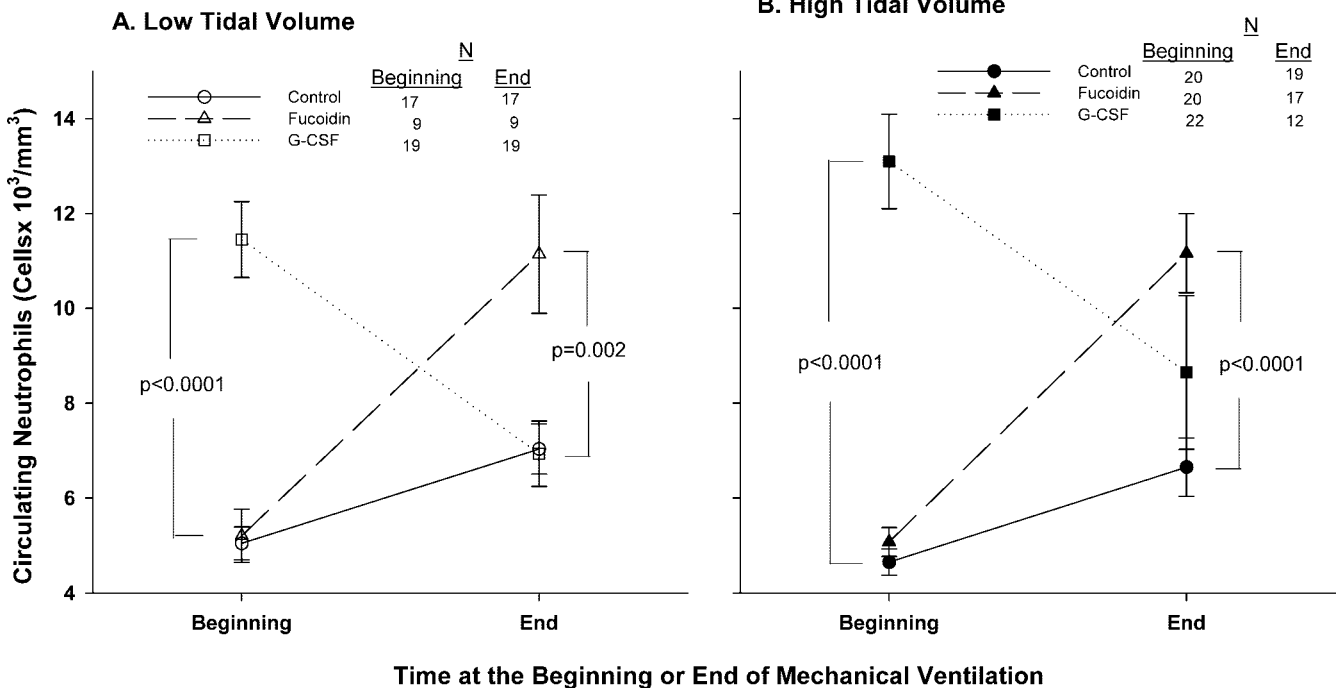


Fig. 2. Mean (\pm SEM) circulating neutrophils at the beginning and end of mechanical ventilation with low (A) or high (B) tidal volume in controls or animals treated with fucoidin or granulocyte colony-stimulating factor (G-CSF). Compared with controls, circulating neutrophils with both low and high tidal volumes were increased with fucoidin at the end but not beginning of mechanical and with G-CSF at the beginning but not the end.

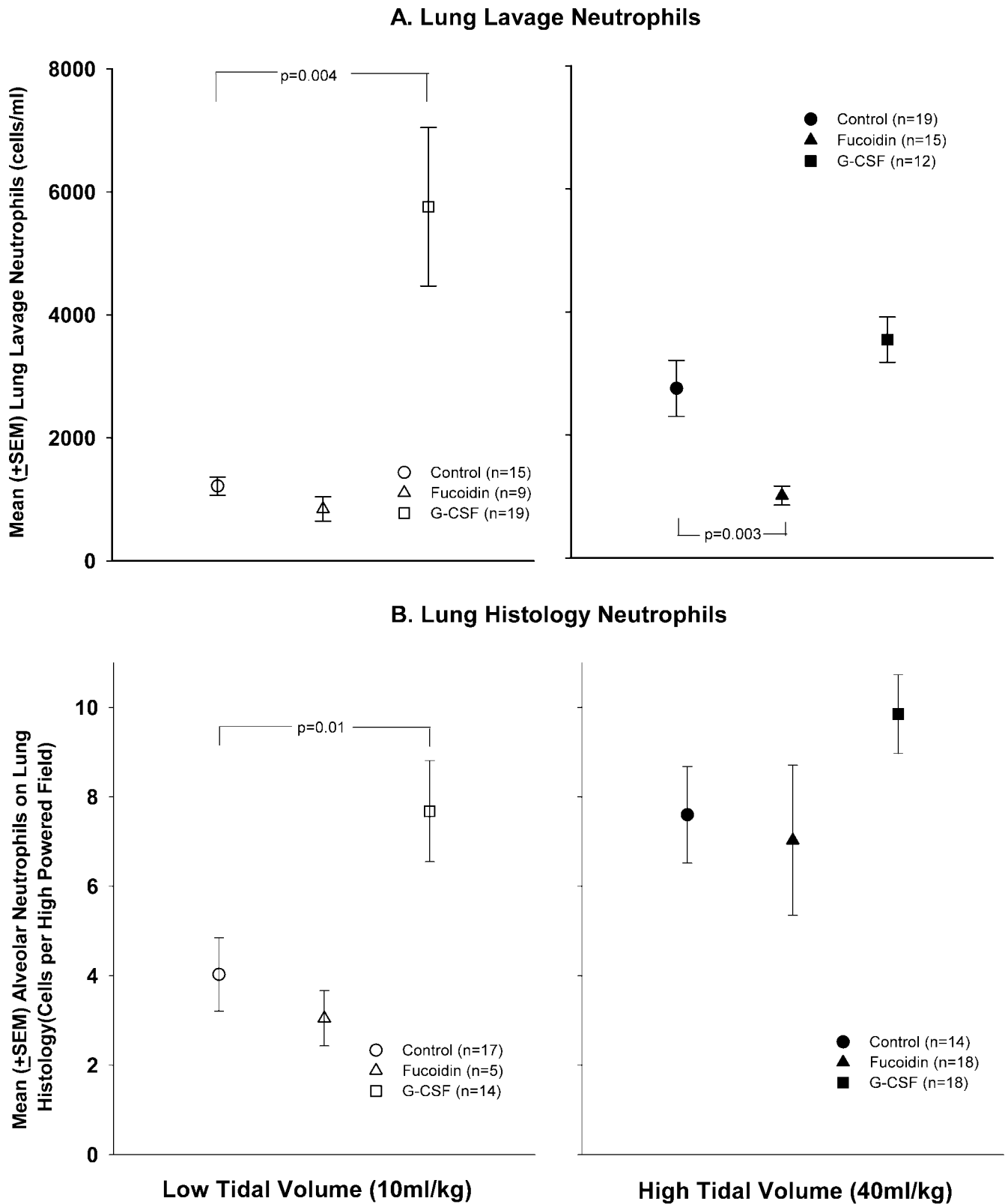


Fig. 3. Mean (\pm SEM) lung lavage neutrophils numbers (cells $\times 10^4$ /ml) (A) and alveolar neutrophil numbers (cells per high-power field) in lung tissue sections (B) at the termination of low- or high-tidal-volume mechanical ventilation in controls or animals treated with fucoidin or granulocyte colony-stimulating factor (G-CSF). Compared with controls, fucoidin significantly reduced lung lavage neutrophils with high tidal volumes, and G-CSF significantly increased both lung lavage and histology neutrophils with low tidal volumes.

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Table 1. Respiratory Parameters from 0 to 4 h after Initiation of Low- (10 ml/kg) or High-tidal-volume (40 ml/kg) Ventilation in Controls or Animals Treated with Fucoidin or G-CSF

Tidal Volume, ml/kg	Treatment	Hours after Initiation of Mechanical Ventilation								
		0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Mean airway pressure, mmHg										
10	Control (n)	4.9 ± 0.2 (17)	4.9 ± 0.1 (17)	5.1 ± 0.2 (17)	5.1 ± 0.1 (17)	5.1 ± 0.2 (17)	5.1 ± 0.1 (17)	5.1 ± 0.1 (17)	4.9 ± 0.2 (17)	5.0 ± 0.1 (17)
	Fucoidin (n)	4.9 ± 0.4 (9)	5.1 ± 0.1 (9)	5.4 ± 0.2 (9)	5.3 ± 0.2 (9)	5.2 ± 0.2 (9)	5.1 ± 0.2 (9)	5.3 ± 0.2 (9)	5.2 ± 0.2 (9)	5.2 ± 0.2 (9)
	G-CSF (n)	4.7 ± 0.2 (20)	4.8 ± 0.2 (20)	4.9 ± 0.2 (20)	5.0 ± 0.2 (20)	5.3 ± 0.2 (20)	5.1 ± 0.2 (20)	5.3 ± 0.2 (20)	5.3 ± 0.2 (20)	5.1 ± 0.2 (20)
40	Control (n)	11.2 ± 0.3 (20)	11.1 ± 0.3 (20)	10.8 ± 0.3 (20)	10.3 ± 0.2 (20)	10.4 ± 0.2 (19)	10.2 ± 0.2 (19)	10.0 ± 0.2 (18)	10.0 ± 0.2 (17)	10.2 ± 0.2 (17)
	Fucoidin (n)	11.6 ± 0.2 (20)	11.2 ± 0.2 (20)	10.9 ± 0.2 (20)	10.5 ± 0.2 (20)	10.5 ± 0.2 (20)	10.4 ± 0.2 (20)	10.1 ± 0.2 (20)	9.9 ± 0.2 (16)	9.9 ± 0.2 (15)
	G-CSF (n)	12.1 ± 0.2 (22)	12.0 ± 0.2 (22)	11.6 ± 0.2 (21)	11.5 ± 0.3 (20)	11.2 ± 0.3 (13)	10.9 ± 0.5 (7)	10.4 ± 0.2 (5)	10.4 ± 0.2 (5)	10.5 ± 0.3 (4)
Arterial oxygen pressure, mmHg										
10	Control	187 ± 12	188 ± 9	196 ± 8	191 ± 9	191 ± 9	203 ± 10	201 ± 6	200 ± 5	200 ± 5
	Fucoidin	193 ± 14	189 ± 11	196 ± 6	189 ± 9	202 ± 15	194 ± 9	201 ± 7	189 ± 8	203 ± 5
	G-CSF	193 ± 12	180 ± 8	181 ± 7	177 ± 7	187 ± 5	181 ± 5	185 ± 5	176 ± 8	185 ± 6
40	Control	197 ± 5	188 ± 5	196 ± 4	196 ± 6	184 ± 8	171 ± 7	164 ± 6	142 ± 12	121 ± 13
	Fucoidin	204 ± 6	204 ± 2	208 ± 6	207 ± 5	198 ± 5	184 ± 7	167 ± 11	177 ± 10	175 ± 7
	G-CSF	201 ± 7	199 ± 5	192 ± 8	169 ± 8	148 ± 12	152 ± 15	137 ± 24	122 ± 20	111 ± 26
Arterial carbon dioxide pressure, mmHg										
10	Control	46 ± 3	44 ± 2	42 ± 2	44 ± 2	45 ± 2	40 ± 2	42 ± 1	40 ± 1	44 ± 2
	Fucoidin	40 ± 3	40 ± 2	41 ± 3	43 ± 2	44 ± 4	41 ± 3	40 ± 4	40 ± 3	40 ± 5
	G-CSF	44 ± 2	46 ± 3	47 ± 3	47 ± 2	47 ± 2	46 ± 2	45 ± 2	44 ± 2	48 ± 2
40	Control	42 ± 1	40 ± 1	40 ± 1	40 ± 1	39 ± 1	39 ± 1	39 ± 1	42 ± 1	42 ± 2
	Fucoidin	39 ± 1	38 ± 1	37 ± 1	38 ± 1	38 ± 1	38 ± 1	37 ± 2	39 ± 1	39 ± 2
	G-CSF	43 ± 3	39 ± 1	40 ± 2	38 ± 1	43 ± 2	41 ± 3	44 ± 2	42 ± 2	46 ± 3

Data are presented as mean ± SEM.

G-CSF = granulocyte colony-stimulating factor; n = animal number observed at each time point, which is the same for all three parameters for each tidal volume and treatment group.

blood pressure ($P = 0.0001$), and heart rate ($P = 0.0001$, ANOVA for all).

Compared with controls, fucoidin did alter any cardiopulmonary parameter significantly with either low or high tidal volumes ($P = \text{NS}$ for all, ANOVA). Compared with controls, from 0 to 4 h, G-CSF with low tidal volumes did not alter airway pressures ($P = \text{NS}$, ANOVA). In contrast, compared with controls, from 0 to 4 h, G-CSF with high tidal volumes was associated with significant increases in airway pressure ($P < 0.0001$ for the effect of G-CSF averaged across time, ANOVA; table 1 and fig. 4). Compared with controls, G-CSF from 0 to 4 h decreased arterial oxygen with low and high tidal volumes, but this was only significant with the former ($P < 0.0001$ averaged across time, ANOVA) and not the latter ($P = \text{NS}$, ANOVA) tidal volume (fig. 4A). However, from 0 to 2 h, when most animals receiving high tidal volumes were still present for study, compared with controls, G-CSF was associated with significant reductions in arterial oxygen with both tidal volumes ($P = 0.02$ for each averaged across time, ANOVA; fig. 5). Compared with controls, G-CSF from 0 to 4 h did not significantly alter systolic arterial blood pressure, heart rate, or arterial carbon dioxide with either tidal volume ($P = \text{NS}$ for all, ANOVA).

Histologic Lung Edema. At the end of mechanical ventilation in controls, compared with low tidal volumes, high ones were associated with significant in-

creases in alveolar edema on lung histology ($P < 0.0001$, ANOVA; fig. 4B). Compared with controls, fucoidin did alter alveolar edema with either low or high tidal volumes ($P = \text{NS}$ for both, ANOVA). Compared with controls, however, G-CSF was associated with significant increases in alveolar edema with both low and high tidal volumes ($P < 0.0001$ and $P = 0.0006$ respectively, ANOVA).

Lung Lavage Protein and Lavage and Circulating Cytokines. At the end of mechanical ventilation in controls, compared with low tidal volumes, high ones were associated with significant increases in lung lavage protein and IL-6 concentrations ($P = 0.02$ and $P < 0.0001$ respectively, ANOVA), but other parameters did not differ significantly (table 2). Compared with controls, fucoidin with high tidal volumes significantly reduced lung lavage IL-6 concentrations ($P < 0.0001$, ANOVA). In contrast, compared with controls, G-CSF significantly increased IL-6 concentrations in blood with low and high tidal volumes ($P = 0.01$ and $P = 0.02$ respectively, ANOVA) and in lung lavage with high tidal volumes ($P = 0.007$, ANOVA). Other protein and cytokine measures did not differ significantly comparing either tidal volume or treatments ($P = \text{NS}$ for all, ANOVA). Other laboratory parameters did not differ comparing either high or low tidal volumes or with either treatment ($P = \text{NS}$ for all, ANOVA; table 2).

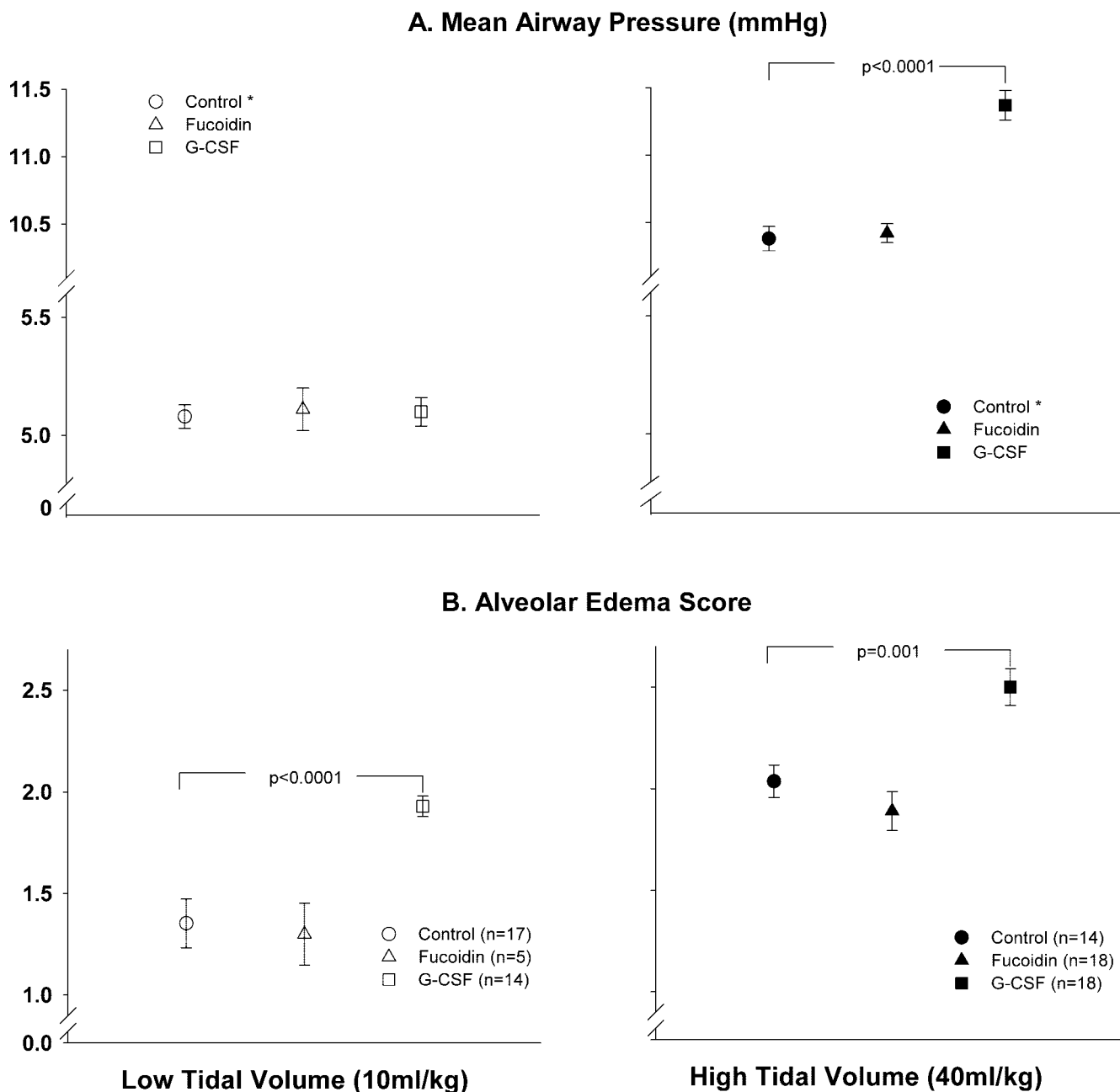


Fig. 4. Mean (\pm SEM) mean airway pressure (mmHg) over the time of mechanical ventilation (A) and mean (\pm SEM) scores for alveolar edema in lung tissue sections at the termination of mechanical ventilation with low or high tidal volumes in controls or animals treated with fucoidin or granulocyte colony-stimulating factor (G-CSF). Compared with controls, G-CSF significantly increased airway pressures with high tidal volumes and increased alveolar edema scores with low and high tidal volumes. * Numbers of animals studied for airway pressure measurements at each time point are shown in table 2.

Discussion

Different from some previous studies that used lung lavage with surfactant depletion in models of VILI, the current one investigated the effects of neutrophil inhibition in a model of VILI related to increases in tidal volumes alone.^{13,14,21} In this study, fucoidin seemed to inhibit lung neutrophil-endothelial adhesion and extravascular migration as evidenced by increases in circulating neutrophils over the time of mechanical ventila-

tion in association with reductions in lung lavage neutrophils and IL-6 concentrations. Despite these changes, fucoidin did not significantly alter lung injury or prevent lethality associated with increased tidal volumes. Absence of a beneficial survival effect with fucoidin is unlikely related to insufficient sample size because this treatment actually increased mortality rates compared with controls. Analysis showed that more than 400 animals would be required divided between placebo

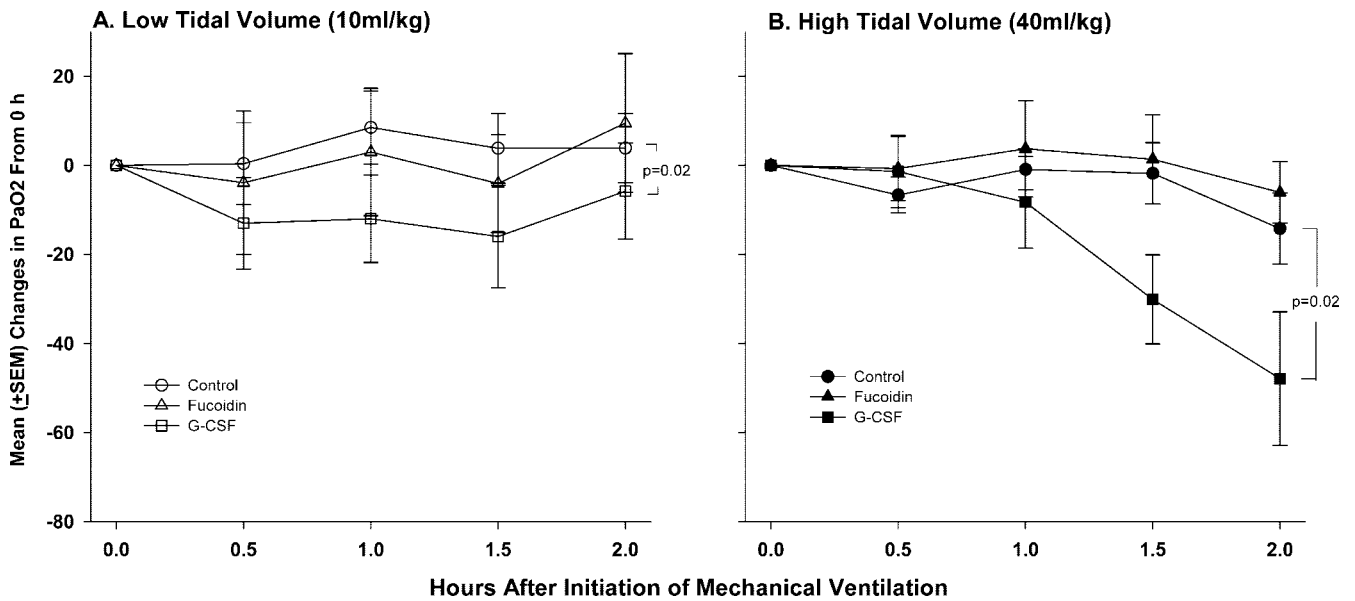


Fig. 5. Mean (\pm SEM) changes in arterial oxygen (mmHg) from 0 to 2 h of mechanical ventilation with low or high tidal volumes in controls or animals treated with fucoidin or granulocyte colony-stimulating factor (G-CSF). Compared with controls, from 0 to 2 h with both low and high tidal volumes, G-CSF resulted in significant decreases in arterial oxygen. Numbers of animals studied for arterial oxygen measurements at each time point are shown in table 2.

and fucoidin groups to demonstrate a significant reversal in the current survival trend with the latter treatment. Similar to the current findings, we observed before that leukocyte integrin adhesion molecule inhibition reduced lung neutrophil recruitment but not mortality rates with lethal hyperoxia.¹⁹ One possible but not conclusive interpretation of these findings with fucoidin is that when unstimulated, selectin-mediated neutrophil recruitment may not have a primary role in lung injury in this model. Alternatively, non-selectin-mediated neutrophil events or mediators independent of the neutrophil may contribute to this injury and the reduced survival rates associated with high tidal volumes in this rat model.²¹⁻²⁶ We cannot exclude the possibility, however, that longer treatment with fucoidin or use of higher doses may have resulted in beneficial effects on either lung injury or survival rates.

In contrast to the negligible effect fucoidin had on

outcome, G-CSF treatment sufficient to increase lung neutrophils and both lung and serum IL-6 concentrations worsened survival rates with high tidal volumes. G-CSF also increased alveolar edema on histology, reduced lung compliance as manifested by increased mean airway pressures, and decreased arterial oxygenation. These effects of G-CSF may relate in part to the sequestration of neutrophils in the pulmonary vasculature that has been shown to occur with unstimulated neutrophils during positive-pressure ventilation in other studies.^{27,28} In the presence of G-CSF, however, more neutrophils than in controls may have been sequestered and then recruited into the alveolar space either because of their increased numbers or level of activity. Marked decreases in circulating neutrophils in G-CSF-treated animals over the course of mechanical ventilation in combination with increases in alveolar neutrophils on tissue sections and in lung lavage are consistent with excessive recruitment

Table 2. Lung Lavage Protein, IL-6, and TNF Concentrations and Serum IL-6 and TNF Concentrations 4 h after Initiation of Low- (10 ml/kg) or High-tidal-volume (40 ml/kg) Ventilation in Controls or Animals Treated with Fucoidin or G-CSF

Tidal Volume, ml/kg	Treatment	Bronchoalveolar Lavage			Serum	
		Protein, μ g/ml	IL-6, pg/ml	TNF, pg/ml	IL-6, pg/ml	TNF, pg/ml
10	Control (n)	102 \pm 19 (16)	40 \pm 10 (17)	10 \pm 5 (18)	35 \pm 12 (17)	196 \pm 146 (18)
	Fucoidin (n)	58 \pm 13 (4)	—	29 \pm 19 (8)	—	118 \pm 30 (8)
	G-CSF (n)	1,431 \pm 1,311 (15)	58 \pm 12 (15)	14 \pm 8 (20)	207 \pm 69 (15)†	595 \pm 456 (20)
40	Control (n)	3,215 \pm 1,385 (14)*	301 \pm 45 (15)*	29 \pm 11 (13)	20 \pm 9 (15)	132 \pm 39 (15)
	Fucoidin (n)	1,630 \pm 740 (16)	58 \pm 17 (16)†	15 \pm 3 (16)	110 \pm 47 (16)	113 \pm 16 (16)
	G-CSF (n)	2,061 \pm 442 (7)	761 \pm 221 (6)†	61 \pm 47 (10)	1,139 \pm 675 (7)†	298 \pm 109 (9)

Data are presented as mean \pm SEM.

* Different from control with low tidal volume, $P < 0.05$. † Different from control at the same level of tidal volume, $P < 0.05$.

G-CSF = granulocyte colony-stimulating factor; IL = interleukin; n = animal number of each parameter performed for each tidal volume and treatment group; TNF = tumor necrosis factor.

of circulating cells. The fact that G-CSF worsened survival, mean airway pressures, and oxygenation with high but not low tidal volumes suggests that high tidal volumes or associated mediators in combination with G-CSF worked synergistically to cause injury. Such an interaction between increased pressure or stretch and leukocyte activity, although not described for the neutrophil, has been demonstrated with endotoxin-activated macrophages *in vitro*.²² However, stimuli other than mechanical ones have been shown to work synergistically with G-CSF to increase the activity and release of inflammatory mediators by neutrophils.²⁹⁻³³ Although G-CSF has been reported to cause systemic capillary leak syndrome and shock, there was no evidence that it aggravated the hypotension associated with high tidal volumes in this rat model.^{34,35}

The absence of increases in serum TNF or IL-6 concentrations in control animals receiving high tidal volumes despite decreased systemic hemodynamic function and survival rates in the current study is in contrast to some but not other investigations.³⁶⁻³⁸ However, high tidal volumes were associated with significant increases in alveolar concentrations of IL-6, a cytokine that has been associated with inflammatory tissue injury and VILI in patients and animal models.¹⁰ Lavage TNF- α was increased as well with high tidal volumes, but not significantly. With longer observation, it is possible that pulmonary production of inflammatory cytokines such as IL-6 or TNF- α might have become evident in the systemic circulation and contributed to hemodynamic instability or nonpulmonary organ injury. To what extent increased lung IL-6 concentrations were the cause as opposed to the result of lung neutrophil recruitment and injury with high tidal volumes is not known. Consistent with the former possibility, reductions in lavage neutrophils with fucoidin were also associated with reductions in IL-6, whereas the opposite was evident with G-CSF. Consistent with the latter, however, in other studies, inhibition of CXC chemokines, another class of inflammatory cytokine, decreased lung neutrophil recruitment and VILI in mice, suggesting that such cytokine production has a direct role in this injury.³⁹ Whether similar chemokines are increased with high tidal volumes in this rat model requires further study. Increased alveolar chemokine production with high tidal volumes in combination with increases in circulating neutrophils could provide a basis for the worsened outcome with G-CSF. Although G-CSF has been reported to both decrease and increase inflammatory cytokines under differing conditions, in the current study, it seemed primarily to have a proinflammatory effect as reflected by the increases in IL-6 it produced.^{40,41} Reductions in IL-6 with fucoidin in the current study are consistent with this agent's inhibition of other antiinflammatory cytokines in previous studies.⁴²

Worsened outcome with G-CSF in this model is concerning given its proposed application in nonneuro-

penic patients with or at risk of pneumonia and sepsis.^{18,43} These patients frequently have development of acute lung injury necessitating mechanical ventilation. Even when tidal volumes are reduced with severe injury, remaining normal lung units may undergo excessive inflation. The findings from our study raise the possibility that G-CSF therapy in patients with advanced lung injury requiring mechanical ventilation could worsen pulmonary dysfunction or even outcome. This danger may be greater with high oxygen concentrations based on studies showing that G-CSF may worsen hyperoxic lung injury.¹⁹ However, G-CSF does have beneficial host defense effects with pneumonia and other types of infection.^{18,44,45} In patients with acute lung injury related to bacterial infection or who are at risk of infection, the beneficial effects of G-CSF on microbial clearance may outweigh any potential adverse effects related to mechanical ventilation. Two large clinical trials have now evaluated the use of G-CSF in patients with severe pneumonia or sepsis, some of whom would have required mechanical ventilation.⁴⁶⁻⁴⁸ Although the incidence of ARDS was increased in each study in patients receiving G-CSF, these increases were not noted to be significant, nor was G-CSF found to be harmful overall in either study.

In summary, although neutrophil inhibition by fucoidin had no measurable effect on reduced survival rates during mechanical ventilation with high tidal volumes in this rat model, neutrophil stimulation with G-CSF worsened them. Extrapolation of these findings clinically suggests that in critically ill patients at risk of VILI, neutrophil activation, either with immunostimulatory agents such as G-CSF or by concurrent conditions such as sepsis, may aggravate that injury. Of note, the increases in circulating neutrophil number noted with G-CSF treatment in the current study are similar in magnitude to the increases noted previously in rats challenged with intrabronchial bacteria.⁴⁹ Whether inhibiting activated neutrophils will be beneficial in ventilated patients at risk of VILI and exposed to such stimuli as G-CSF or sepsis requires further study.

References

1. Dos Santos CC, Slutsky AS: Invited review: Mechanisms of ventilator-induced lung injury: A perspective. *J Appl Physiol* 2000; 89:1645-55
2. Plotz FB, Slutsky AS, van Vught AJ, Heijnen CJ: Ventilator-induced lung injury and multiple system organ failure: A critical review of facts and hypotheses. *Intensive Care Med* 2004; 30:1865-72
3. The Respiratory Distress Syndrome Network: Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. *N Engl J Med* 2000; 342:1301-8
4. Parker JC, Hernandez LA, Peevy KJ: Mechanisms of ventilator-induced lung injury. *Crit Care Med* 1993; 21:131-43
5. Uhlig S: Ventilation-induced lung injury and mechanotransduction: Stretching it too far? *Am J Physiol Lung Cell Mol Physiol* 2002; 282:L892-6
6. Matsuoka T, Kawano T, Miyasaka K: Role of high-frequency ventilation in surfactant-depleted lung injury as measured by granulocytes. *J Appl Physiol* 1994; 76:539-44
7. Sugiura M, McCulloch PR, Wren S, Dawson RH, Froese AB: Ventilator

pattern influences neutrophil influx and activation in atelectasis-prone rabbit lung. *J Appl Physiol* 1994; 77:1355-65

8. Tsuno K, Miura K, Takeya M, Kolobow T, Morioka T: Histopathologic pulmonary changes from mechanical ventilation at high peak airway pressures. *Am Rev Respir Dis* 1991; 143:1115-20

9. Woo SW, Hedley-Whyte J: Macrophage accumulation and pulmonary edema due to thoracotomy and lung over inflation. *J Appl Physiol* 1972; 33:14-21

10. Ranieri VM, Suter PM, Tortorella C, De Tullio R, Dayer JM, Brienza A, Bruno F, Slutsky AS: Effect of mechanical ventilation on inflammatory mediators in patients with acute respiratory distress syndrome: A randomized controlled trial. *JAMA* 1999; 282:54-61

11. Downey GP, Dong Q, Kruger J, Dedhar S, Cherapanov V: Regulation of neutrophil activation in acute lung injury. *Chest* 1999; 116:46S-54S

12. Zhang H, Downey GP, Suter PM, Slutsky AS, Ranieri VM: Conventional mechanical ventilation is associated with bronchoalveolar lavage-induced activation of polymorphonuclear leukocytes: A possible mechanism to explain the systemic consequences of ventilator-induced lung injury in patients with ARDS. *ANESTHESIOLOGY* 2002; 97:1426-33

13. Kawano T, Mori S, Cybulsky M, Burger R, Ballin A, Cutz E, Bryan AC: Effect of granulocyte depletion in a ventilated surfactant-depleted lung. *J Appl Physiol* 1987; 62:27-33

14. Rimensberger PC, Fedorko L, Cutz E, Bohn DJ: Attenuation of ventilator-induced acute lung injury in an animal model by inhibition of neutrophil adhesion by leumedins (NPC 15669). *Crit Care Med* 1998; 26:548-55

15. Ley K, Linnemann G, Meinen M, Stoolman LM, Gachtgens P: Fucoidin, but not yeast polyphosphomannan PPME, inhibits leukocyte rolling in venules of the rat mesentery. *Blood* 1993; 81:177-85

16. Linnemann G, Reinhart K, Parade U, Philipp A, Pfister W, Straube E, Karzai W: The effects of inhibiting leukocyte migration with fucoidin in a rat peritonitis model. *Intensive Care Med* 2000; 26:1540-6

17. Shimaoka M, Ikeda M, Iida T, Taenaka N, Yoshiya I, Honda T: Fucoidin, a potent inhibitor of leukocyte rolling, prevents neutrophil influx into phorbol-ester-induced inflammatory sites in rabbit lungs. *Am J Respir Crit Care Med* 1996; 153:307-11

18. Dale DC, Liles WC, Summer WR, Nelson S: Review: Granulocyte colony-stimulating factor-role and relationships in infectious diseases. *J Infect Dis* 1995; 172:1061-75

19. Freeman BD, Correa R, Karzai W, Natanson C, Patterson M, Banks S, Fitz Y, Danner RL, Wilson L, Eichacker PQ: Controlled trials of rG-CSF and CD11b-directed MAb during hyperoxia and *E. coli* pneumonia in rats. *J Appl Physiol* 1996; 80:2066-76

20. Dawson JD, Lagakos SW: Size and power of two-sample tests of repeated measures data. *Biometrics* 1993; 49:1022-32

21. Dreyfuss D, Soler P, Basset G, Saumon G: High inflation pressure pulmonary edema: Respective effects of high airway pressure, high tidal volume, and positive end-expiratory pressure. *Am Rev Respir Dis* 1988; 137:1159-64

22. Pugin J, Dunn I, Jolliet P, Tassaux D, Magnenat JL, Nicod LP, Chevrolet JC: Activation of human macrophages by mechanical ventilation in vitro. *Am J Physiol* 1998; 275:L1040-50

23. Pugin J: Molecular mechanisms of lung cell activation induced by cyclic stretch. *Crit Care Med* 2003; 31:S200-6

24. Vlahakis NE, Schroeder MA, Limper AH, Hubmayr RD: Stretch induces cytokine release by alveolar epithelial cells in vitro. *Am J Physiol* 1999; 277:L167-73

25. Ricard JD, Dreyfuss D, Saumon G: Ventilator-induced lung injury. *Curr Opin Crit Care* 2002; 8:12-20

26. Kolobow T, Moretti MP, Fumagalli R, Mascheroni D, Prato P, Chen V, Joris M: Severe impairment in lung function induced by high peak airway pressure during mechanical ventilation: An experimental study. *Am Rev Respir Dis* 1987; 135:312-5

27. Loick HM, Wendt M, Rotker J, Theissen JL: Ventilation with positive end-expiratory airway pressure causes leukocyte retention in human lung. *J Appl Physiol* 1993; 75:301-6

28. Markos J, Doerschuk CM, English D, Wiggs BR, Hogg JC: Effect of positive end-expiratory pressure on leukocyte transit in rabbit lungs. *J Appl Physiol* 1993; 74:2627-33

29. Khwaja A, Carver JE, Linch DC: Interactions of granulocyte-macrophage

colony-stimulating factor (CSF), granulocyte CSF, and tumor necrosis factor alpha in the priming of the neutrophil respiratory burst. *Blood* 1992; 79:745-53

30. Mur E, Zabernigg A, Hilbe W, Eisterer W, Halder W, Thaler J: Oxidative burst of neutrophils in patients with rheumatoid arthritis: Influence of various cytokines and medication. *Clin Exp Rheumatol* 1997; 15:233-7

31. Wiltshcke C, Kraimer M, Nanut M, Wagner A, Linkesch W, Zielinski CC: In vivo administration of granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor increases neutrophil oxidative burst activity. *J Interferon Cytokine Res* 1995; 15:249-53

32. Yagisawa M, Yuo A, Kitagawa S, Yazaki Y, Togawa A, Takaku F: Stimulation and priming of human neutrophils by IL-1 alpha and IL-1 beta: complete inhibition by IL-1 receptor antagonist and no interaction with other cytokines. *Exp Hematol* 1995; 23:603-8

33. Yuo A, Kitagawa S, Kasahara T, Matsushima K, Saito M, Takaku F: Stimulation and priming of human neutrophils by interleukin-8: Cooperation with tumor necrosis factor and colony-stimulating factors. *Blood* 1991; 78:2708-14

34. de Azevedo AM, Goldber TB: Life-threatening capillary leak syndrome after G-CSF mobilization and collection of peripheral blood progenitor cells for allogeneic transplantation. *Bone Marrow Transplant* 2001; 28:311-2

35. Rechner I, Brito-Babapule F, Fielden J: Systemic capillary leak syndrome after G-CSF. *Hematol J* 2003; 4:54-56

36. Chiumello D, Pristine G, Slutsky AS: Mechanical ventilation affects local and systemic cytokines in an animal model of acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1999; 160:109-16

37. Ricard JD, Dreyfuss D, Saumon G: Production of inflammatory cytokines in ventilator-induced lung injury: A reappraisal. *Am J Respir Crit Care Med* 2001; 163:1176-80

38. von Bethmann AN, Brasch F, Nusing R, Vogt K, Volk HD, Muller KM, Wendel A, Uhlig S: Hyperventilation induces release of cytokines from perfused mouse lung. *Am J Respir Crit Care Med* 1998; 157:263-72

39. Belperio JA, Keane MP, Burdick MD, Londhe V, Xue YY, Li K, Phillips RJ, Strieter RM: Critical role for CXCR2 and CXCR2 ligands during the pathogenesis of ventilator-induced lung injury. *J Clin Invest* 2002; 110:1703-16

40. Valente JF, Alexander JW, Li BG, Noel JG, Custer DA, Ogle JD, Ogle CK: Effect of *in vivo* infusion of granulocyte colony-stimulating factor on immune function. *Shock* 2002; 17:23-9

41. Azoulay E, Attalah H, Yang K, Herigault S, Joullet H, Brun-Buisson C, Brochard I, Harf A, Schlemmer B, Delclaux C: Exacerbation with granulocyte colony-stimulating factor of prior acute lung injury during neutropenia recovery in rats. *Crit Care Med* 2003; 31:157-65

42. Granert C, Raud J, Waage A, Lindquist L: Effects of polysaccharide fucoidin on cerebrospinal fluid IL-1 and TNF α in pneumococcal meningitis in the rabbit. *Infect Immun* 1999; 67:2071-4

43. Azoulay E, Delclaux C: Is there a place for granulocyte colony-stimulating factor in non-neutropenic critically ill patients? *Intensive Care Med* 2004; 30:10-7

44. Eichacker PQ, Waisman Y, Natanson C, Farese A, Hoffman WD, Banks SM, MacVittie TJ: Cardiopulmonary effects of granulocyte colony-stimulating factor in a canine model of bacterial sepsis. *J Appl Physiol* 1994; 77:2366-73

45. Freeman BD, Quezada Z, Zeni F, Natanson C, Danner RL, Banks S, Quezada M, Fitz Y, Bacher J, Eichacker PQ: rG-CSF reduces endotoxemia and improves survival during *E. coli* pneumonia. *J Appl Physiol* 1997; 83:1467-75

46. Nelson S, Albrecht MH, Stone J, Bergerson MG, Daugherty S, Peterson G, Fotherington N, Welch W, Milwee S, Root R: A randomized controlled trial of filgrastim for the treatment of hospitalized patients with multilobar pneumonia. *J Infect Dis* 2000; 182:970-3

47. Parent C, Eichacker PQ: Granulocyte colony stimulating factor as therapy for pneumonia and sepsis in the nonneutropenic host: Preclinical and clinical trials. *Evolving Concepts in Sepsis and Septic Shock*. Edited by Eichacker PQ, Pugin J, Boston, Kluwer Academic Publishers, 2001, pp 175-87

48. Root RK, Lodato RF, Patrick W, Cade JF, Fotherington N, Milwee S, Vincent JL, Torres A, Rello J, Nelson S, Pneumonia Sepsis Study Group: Multi-center, double-blind, placebo-controlled study of the use of filgrastim in patients hospitalized with pneumonia and severe sepsis. *Crit Care Med* 2003; 31:367-73

49. Karzai W, Cui X, Mehlhorn B, Straube E, Hartung T, Gerstenberger E, Banks S, Natanson C, Reinhart K, Eichacker PQ: Protection with antibody to tumor necrosis factor differs with similarly lethal *E. coli* versus *S. aureus* pneumonia in rats. *ANESTHESIOLOGY* 2003; 99:1377-82