Cytokine release, small airway injury, and parenchymal damage during mechanical ventilation in normal open-chest rats

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ABSTRACT

Lung morpho-functional alterations and inflammatory response to various types of mechanical ventilation (MV) have been assessed in normal, anestetized, open-chest rats. Measurements were taken during protective MV (V_T=8 ml·kg^{-1}; PEEP=2.6 cm H_2O) before and after a 2-2½ hours period of ventilation on PEEP (Control group), zero EEP without (ZEEP group) or with administration of dioctylsodiumsulfosuccinate (ZEEP-DOSS group), on negative EEP (NEEP group), or with large V_T (26 ml·kg^{-1}) on PEEP (Hi-V_T group). No change in lung mechanics occurred in the Control group. Relative to the initial period of MV on PEEP, airway resistance increased by 33±4, 49±9, 573±84 and 13±4%, and quasi-static elastance by 19±3, 35±7, 248±12, and 20±3% in the ZEEP, NEEP, ZEEP-DOSS, and Hi-V_T groups. Relative to Control, all groups ventilated from low lung volumes exhibited histologic signs of bronchiolar injury, more marked in the NEEP and ZEEP-DOSS groups. Parenchymal and vascular injury occurred in the ZEEP-DOSS and Hi-V_T groups. Pro-inflammatory cytokine concentration in the bronchoalveolar lavage fluid (BALF) was similar in the Control and ZEEP group, but increased in all other groups, and higher in the ZEEP-DOSS and Hi-V_T groups. Rint was correlated with indices of bronchiolar damage, and cytokine levels with vascular-alveolar damage, as indexed by lung wet-to-dry ratio. Hence, protective MV from resting lung volume causes mechanical alterations and small airway injury, but no cytokine release, which seems mainly related to stress-related damage of endothelial-alveolar cells. Enhanced small airway epithelial damage with induced surfactant dysfunction or MV on NEEP can however contribute to cytokine production.

keywords: lung mechanics, recruitment-derecruitment of lung units, lung injury, microvascular damage, inflammation

In an *ex vivo* model of normal and lavaged rat lungs, ventilation with physiologic tidal volumes (V_T) from zero end-expiratory pressure (ZEEP) causes mechanical alterations with a significant increase of histologic injury scores in the terminal bronchioles, and release of pro-inflammatory cytokines (5, 19, 24). Subsequently, it was shown that also in normal, anesthetized rabbits, mechanical ventilation at low lung volumes induces histological evidence of peripheral airway damage with a concurrent increase in airway resistance and lung elastance, which persist after restoration of normal end-expiratory volumes (6-8), and that these effects are caused by the abnormal stresses due to cyclic opening and closing of peripheral airways and increased surface tension (9). In contrast with the *in vitro* studies on rats, in the *in vivo* studies on rabbits there was no indication of an inflammatory response as assessed by the release of inflammatory cytokines, further suggesting that the so called "low volume injury" should be due to the mechanical stress related to cyclic opening and closing of small airways, hereafter referred to as tidal airway closure.

The discrepancy in the release of inflammatory cytokines could be, however, inherent to the models, i.e. *in vivo* vs *in vitro* preparation. Differences in the extension of small airway involvement in tidal airway closure or degree of surfactant dysfunction and dependent noxious stress also provide alternative explanations. On the other hand, the discrepancy could be simply apparent: indeed, only tumor necrosis factor-alfa (TNF-α) was assessed in rabbits, and although the largest changes usually affect this cytokine (5, 25), it has been shown that release of pro-inflammatory cytokines can occur with little or no change in TNF-α levels (26).

The aim of this study is that of establishing whether tidal airway closure with ventilation at low volumes induces an inflammatory response with release of inflammatory cytokines in normal lungs *in vivo*. The concentration of the pro-inflammatory cytokines, and the concomitant functional and histologic alterations were, therefore, assessed in normal, anesthetized open-chest rats ventilated with physiological V_T on ZEEP. In order to increase the amount of small airways involved in tidal airway closure and/or stress associated with this phenomenon, a group of open-chest rats was mechanically ventilated with physiological V_T on negative end-expiratory pressure (NEEP), while another group was ventilated on ZEEP after having been treated with the aerosolized detergent dioctylsodiumsulfosuccinate to increase surface tension. Measurements were also obtained from a fourth group of open-chest rats ventilated with large V_T on positive end-expiratory pressure (PEEP), because it is generally recognized that release of cytokines invariably occurs under this condition as a consequence of parenchymal overstretching (10,12). Finally, measurements were performed in normal, untreated, open-chest rats subjected to "non injurious" ventilation, i.e. prolonged mechanical ventilation with physiological V_T on PEEP, these animals serving as the control group.

METHODS

Thirty-five male Sprague-Dawley rats (weight range 380-460 g) were anesthetized with an intraperitoneal injection of a mixture of pentobarbital sodium (40 $mg \cdot kg^{-1}$) and chloral hydrate (170 $mg \cdot kg^{-1}$), after induction with diazepam (10 $mg \cdot kg^{-1}$). A metal cannula, connected to a pneumotachograph, and two polyethylene catheters were inserted into the trachea, jugular vein, and carotid artery, respectively. The animals were paralyzed with pancuronium bromide (0.1 $mg \cdot kg^{-1}$), and ventilated with a pattern similar to that during spontaneous breathing using a custom made ventilator. Anesthesia and complete muscle relaxation were maintained with additional doses of the anesthetic mixture and pancuronium bromide. Adequacy of anesthesia was judged from the sudden increase in heart rate and/or systemic blood pressure. The chest was opened via a median sternotomy and a coronal cut made just above the costal arch, while application of positive end-expiratory pressure (PEEP) prevented lung collapse.

Airflow ($\dot{V}$) was measured with a heated Fleisch pneumotachograph no.0000 (HS Electronics, March-Hugstetten, Germany) connected to the tracheal cannula and a differential pressure transducer (Validyne MP45, ±2 cmH_2O; Northridge, CA). The response of the pneumotachograph was linear over the experimental flow range. Tracheal pressure (Ptr) and systemic blood pressure were measured with pressure transducers (8507C-2 Endevco, San Juan Capistrano, CA; Statham P23Gb, HS Electronics, March-Hugstetten, Germany) connected to the side arm of the tracheal cannula and carotid catheter, respectively. There was no appreciable shift in the signal or alteration in amplitude up to 20 Hz. The signals from the transducers were amplified (RS3800; Gould Electronics, Valley View, OH), sampled at 200 Hz by a 12-bit A/D converter (AT MIO 16L-9; National Instruments, Austin, TX), and stored on a desk computer. Volume changes (ΔV) were obtained by numerical integration of the digitized airflow signal. Arterial blood P_{O_2}, P_{CO_2} and pH were measured by means of a blood gas analyzer (IL 1620; Instrumentation Laboratory, Milan, Italy) on samples drawn at the end of each test session.

After completion of the surgical procedure, the rats were ventilated with a specially designed, computer-controlled ventilator (6), delivering water-saturated air from a high pressure source (4 atm) at constant flow of different selected magnitudes and duration, while Ringer-bicarbonate was continuously infused intravenously at a rate of 4 $ml \cdot kg^{-1} \cdot h^{-1}$, and epinephrine occasionally administered to keep normal arterial blood pressure. A three way stopcock allowed the connection of the expiratory valve of the ventilator either to the ambient (ZEEP) or to a drum in which the pressure was set at 2.5-2.8 (PEEP) or –3 cmH2O (NEEP) by means of a flow-through system. While on PEEP air was used to ventilate the animals, on ZEEP and NEEP oxygen (70-

80%) mixtures were intermittently administered if needed to prevent marked, life threatening hypoxia. However, all measurements were always performed during air breathing. Baseline ventilation consisted of a fixed V_T (8 ml·kg^{-1}), inspiratory and expiratory duration (0.25 and 0.5 s), and end-inspiratory pause (0.2 s). No intrinsic PEEP was present under any experimental condition, as evidenced by the absence of Ptr changes with airway occlusion at end-expiration. During measurements, the ribs on the two sides and the diaphragm were pulled widely apart, in order to prevent contact between lung and chest wall, except in their dependent parts.

Surfactant dysfunction was induced by means of 10% alcoholic solution of dioctylsodium-sulfosuccinate (DOSS), (Aerosol OT, A 6627; Sigma-Aldrich, St. Louis, MO) diluted 1:2 in saline. The aerosolized 5% DOSS solution was delivered by a nebulizer (Ultra-Neb 99; DeVilbiss, Somerset, PA) for 90 consecutive inflations. Rats were discarded in which DOSS administration on PEEP caused a marked increase in elastance, grossly inhomogeneous lung expansion, and, occasionally, overt edema.

Procedure and data analysis

All rats underwent an initial ($PEEP_1$) and final 30 min period ($PEEP_2$) of baseline ventilation on PEEP (2.6±0.02 cmH_2O), separated by a 2-2.5 h period during which one of the following ventilation types was used: *a)* baseline ventilation on ZEEP (ZEEP group); *b)* baseline ventilation on NEEP of –3 cmH_2O (NEEP group); *c)* baseline ventilation on ZEEP after treatment with DOSS (ZEEP-DOSS group); *d)* high volume (V_T=26 ml·kg^{-1}) ventilation on PEEP (Hi-V_T group); and *e)* baseline ventilation on PEEP (Control group). When large V_T were used, the inspiratory (T_I) and expiratory duration were increased (1 and 2.9 s, respectively) in order to keep pulmonary ventilation nearly constant. Each group was made of 7 animals, and the various types of experiment were done in random order.

Lung mechanics was assessed during the $PEEP_1$ and $PEEP_2$ periods, and at the end of the period of test ventilation. Two types of measurements were carried out: *a)* while keeping V_T at baseline values, test breaths were intermittently performed with different $\dot{V}_I$ and T_I in the range 0.25 to 3 s to assess lung mechanics at end-inflation; and *b)* while keeping $\dot{V}_I$ constant, test breaths were intermittently performed with different V_T to obtain the quasi-static inflation volume-pressure curve in the tidal volume range. End-inspiratory occlusions lasting 5 s were made in all test breaths, and repeated 4-5 times under each experimental condition. On PEEP, the lungs were inflated 3-4 times to Ptr of ~25 cmH_2O before all measurements, and the expiratory valve was opened to the ambient for 3-5 expirations in order to measure the difference between the end-expiratory and the resting lung volume (ΔEELV), the latter being the volume at zero transpulmonary pressure.

Quasi static elastance (E_{st}), interrupter resistance (R_{int}), which reflects airway resistance, and viscoelastic resistance (R_{visc}) and time constant (τ_{visc}) were assessed according to the rapid airway occlusion method, as previously described (6), while the ratio (E_{low}/E_{base}) between E_{st} with the lowest V_T (~1.3 ml·kg^{-1}) and baseline V_T was taken as an index of the amount of peripheral airways being involved in recruitment-derecruitment during tidal ventilation (9,15).

After completion of the mechanics measurements, 1.5–2 ml of blood were drawn from the heart for the assessment of systemic release of cytokines and serum albumin concentration. The animals were killed with an overdose of anesthetics. The right lung was processed for histological analysis (see below). The main left bronchus was cannulated, the left lung removed, weighed immediately, lavaged with 4.3 ml·kg^{-1} of normal saline in two aliquots, fluid recovery ranging from 40 to 50%, left overnight in an oven at 120 °C, and weighed again to compute the wet-to-dry ratio (W/D). The effluents were pooled, centrifuged (Harrier 18/80, Sanyo Gallenkamp PLC, Loughborough, UK) at 2000 rpm for 10 min, and the supernatant frozen and stored at –20°C, for subsequent assessment of cytokines and albumin concentration in broncho-alveolar lavage fluid (BALF).

Cytokine (TNF-α, IL-1β, IL-2, IL-6, IL-10, MIP-2) analysis was carried out in duplicate in blinded fashion on BALF and serum using commercially available ELISA kits specific for rat (Quantikine, R&D Systems, Inc., Minneapolis, MN; Rat GRO/CINC-3 Assay Kit, IBL, Japan). Absorbance was read at 450 nm (correction wavelength set at 540 nm) (Titertek Multiskan MCC, Flow Laboratories, Milan, Italy), background absorbancy of blank wells being subtracted from the standards and samples prior to determination of the concentration. The lower limit of detection for those kits was 6.25, 15.6, 15.6, 62.5, 31.2, and 5 pg·ml^{-1}, respectively, in which case concentration was assumed to be nil. The albumin concentration of the BALF supernatant and serum obtained shortly before lung lavage was determined with a clinical chemistry analyzer (Bayer ADVIA 2004, Jeol, Japan for Bayer Diagnostics Europe, Dublin, Ireland) at 596 nm using the BCG method (Albumin reagent, Bayer, Tarrytown, UK) with bovine albumin as standard.

Histological analysis

The right lung was fixed by intratracheal infusion of a 8% formaldehyde, 0.1% glutaraldehyde solution with the pressure maintained at 20 cmH_2O for 24 h. Three blocks, ~1 cm thick, involving both subpleural and para-hilar regions, were obtained in each animal. Each block was processed through a graded series of alcohols and embedded in paraffin. From each block, sections of 5 μm thickness were cut and stained with hematoxylin-eosin. Histologic evaluation was performed by a single observer in a blind fashion, according to the procedure previously described in details (6, 7, 9). The following measures were obtained using a computer-aided, image analysis

system (IMAQ Vision for LabView; National Instruments, Austin, TX): *a)* the percent ratio of lesioned (epithelial necrosis and sloughing) to total membranous bronchioles, the bronchiolar injury score (IS), as an index of small airway injury (19); and *b)* the percent ratio of abnormal to total (normal and abnormal) bronchiolar-alveolar attachments, and the distance between normal attachments, as an indices of airway-parenchymal mechanical uncoupling.

In addition, parenchymal and vascular injury was assessed by four parameters, focal alveolar collapse, perivascular and/or alveolar edema, recruitment of granulocytes to the air spaces, and hemorrhage (24), evaluated semiquantitatively with a four-grade scale (absent=0; mild=1; moderate=2; marked=3).

The study, which conforms to the American Physiological Society's guidelines for animal care, was approved by Ministero della Salute, Rome, Italy.

Statistics.

Analyses were performed using SPSS 11.5 (SPSS Inc., Chicago, IL). Results from mechanical studies are presented as means±SE. Comparisons among experimental conditions were performed using one-way analysis of variance (ANOVA); when significant differences were found, the Bonferroni correction was made to determine significant differences between different experimental conditions. Results from cytokine assessments and histological studies are expressed as median and range, and the statistical analysis was done using the Mann-Whitney test. Multiple linear regression analysis was performed according to the least mean square method. The level for statistical significance was taken at $P \leq 0.05$.

RESULTS

Blood gasses and pH

During the initial period of ventilation on PEEP ($PEEP_1$), the mean values of arterial P_{O_2}, P_{CO_2}, and pH were similar for all groups of rats (Fig. 1). With ventilation at low end-expiratory lung volume, pHa decreased significantly in all groups, Pa_{O_2} decreased in the NEEP and ZEEP-DOSS group, while Pa_{CO_2} increased only in the ZEEP-DOSS group. On $PEEP_2$, pHa was significantly reduced relative to $PEEP_1$ values by the same amount in all groups of animals. While Pa_{CO_2} returned to the initial values on $PEEP_1$ in all groups, Pa_{O_2} was significantly decreased only in animals treated with DOSS.

On $PEEP_1$, the mean systemic blood pressure was similar in all groups of rats, averaging 80±2 mmHg. It decreased during ventilation both with physiological V_T from low lung volumes (-11±4 mmHg) and with large V_T from physiological lung volumes (-6±4 mmHg), likely because of

increased pulmonary vascular resistance and reduced left atrial filling. No significant differences in mean systemic blood pressure occurred between $PEEP_1$ and $PEEP_2$ in all groups.

Mechanics

During $PEEP_1$, no significant differences occurred among the various groups for any mechanical parameter. Apart from a small non-significant increase of lung elastance, DOSS administration had no mechanical effects.

Ventilation at low EELV increased R_{int}, E_{st}, and R_{visc} in all groups of animals, while τ_{visc} decreased significantly in the NEEP and ZEEP-DOSS group only (Fig. 2). Relative to $PEEP_1$, R_{int} increased by 533±53, 769±98, and 1035±186%, E_{st} by 319±14, 363±35, and 449±31%, and R_{visc} by 118±12, 142±26, and 212±16% in the ZEEP, NEEP, and ZEEP-DOSS group, respectively. The quasi-static inflation V-P curve (Fig. 3), which on PEEP was concave towards the pressure axis, became sigmoid in the ZEEP and NEEP group, and convex towards the pressure axis in the ZEEP-DOSS group. As a consequence, E_{low}/E_{base} increased markedly during ventilation at low EELV (Fig. 2), and significantly more in the ZEEP-DOSS and NEEP group than in the ZEEP group: to the extent that the increase in E_{low}/E_{base} reflects tidal recruitment of lung units, the latter should have been larger in the ZEEP-DOSS than in the NEEP and ZEEP group. Indeed, cardiac artifacts in the Ptr records were always present during the occlusion at end-inspiration, but absent at end-expiration in four and two animals of only the ZEEP-DOSS and NEEP group, respectively.

With the same end-expiratory Ptp (2.6±0.2 cm H_2O), the EELV was similar on $PEEP_1$ and $PEEP_2$, except in the ZEEP-DOSS group where it decreased from 3±0.2 to 1.8±0.2 ml (Fig. 3). The inflation V-P in the V_T range resumed the initial shape in all groups: as a consequence, E_{low}/E_{base} did not differ significantly between $PEEP_1$ and $PEEP_2$ (Fig. 2).

Prolonged ventilation on PEEP had no mechanical effects in animals ventilated with physiological V_T (Control group), while in those ventilated with large V_T (Hi-V_T group) Rint and E_{st} were significantly increased (13±3 and 20±3%; $P<0.001$). After restoration of PEEP in animals previously ventilated at low EELV, R_{int} and E_{st} remained elevated in all groups, R_{visc} only in animals treated with DOSS, while τ_{visc} returned to control values in all groups (Fig. 2). Relative to $PEEP_1$, R_{int} increased by 33±4, 49±9, and 573±84% and E_{st} by 19±3, 35±7, and 248±12% in the ZEEP, NEEP, and ZEEP-DOSS group, respectively, while R_{visc} increased by 79±15% in the ZEEP-DOSS group.

Cytokines

Fig. 4 shows the absolute levels of inflammatory and anti-inflammatory cytokines in serum and BALF for the various groups of rat, IL-2 concentration being below detectable levels under all circumstances. The lowest levels of inflammatory cytokines were found in the Control group. In the

ZEEP group, serum and BALF concentration of inflammatory cytokines did not differ significantly from that in the Control group. In the other groups of rats the concentration of inflammatory cytokines in BALF was significantly higher than that of the Control group, while that of the anti-inflammatory citokine IL-10 was significantly increased in the ZEEP-DOSS group only. TNF-α levels did not differ among the NEEP, ZEEP-DOSS, and Hi-V_T groups, whereas the concentration of MIP-2, IL-1β, and IL-6 was significantly higher in the ZEEP-DOSS than in the NEEP group, but similar in the ZEEP-DOSS and Hi-V_T group. In general, cytokine levels in serum paralleled those in BALF: except for TNF-α, there was in fact a significant correlation between serum and BALF concentrations (Fig. 5).

Histology

Scores of bronchiolar epithelial injury (IS) and airway-parenchymal uncoupling (% abnormal bronchiolar-alveolar attachments, distance between normal attachments) are shown in Table 1 for all groups. Epithelial injury of small airways and airway-parenchymal uncoupling, i.e. abnormal bronchiolar-alveolar attachments, were more prominent in rats subjected to mechanical ventilation at low lung volume than in those ventilated on PEEP only, the injury score being in turn significantly higher in the NEEP and ZEEP-DOSS groups, in which it was similar, than in the ZEEP group. Some degree of airway-parenchymal uncoupling occurred, however, in the Hi-V_T group too, as the number of abnormal bronchiolar-alveolar attachments, but not the distance between normal attachments, was significantly higher than that in the Control group. Damage of cartilaginous airways was never observed.

Scores of parenchymal and vascular injury are shown in Table 2. No parenchymal and vascular damage occurred in rats of the Control and ZEEP group, while in the NEEP group one animal showed mild focal alveolar collapse, two animals recruitment of granulocytes to the air space, and three animals mild to moderate perivascular and peribronchial edema. In contrast, the lungs of all animals in the ZEEP-DOSS and Hi-V_T group were presenting various combinations of parenchymal and vascular injuries, that were more prominent in rats treated with DOSS, particularly interstitial and alveolar edema.

Measurements of lung wet-to-dry (W/D) ratio and ratio of BALF to serum albumin concentration (A_{BALF}/A_{SER}) reported in Table 2 further support the morphologic evaluation of lung edema. The W/D ratio was similar in the Control, and ZEEP group, higher, though not significantly, in the NEEP group, and significantly increased in the Hi-V_T and even more in the ZEEP-DOSS group. The A_{BALF}/A_{SER} ratio was also similar in the Control, ZEEP, and NEEP group; relative to Control group values, it was significantly increased in the Hi-V_T and even more in the ZEEP-DOSS group, indicating that alveolar edema developed mainly in the latter group.

DISCUSSION

In normal, open-chest rats prolonged mechanical ventilation with physiologic end-expiratory and tidal volumes (PEEP group) did not cause mechanical changes (Figures 2 and 3) and essentially no signs of lung injury (Tables 1 and 2), in line with the results obtained in normal rabbits (6,7). On the other hand, prolonged mechanical ventilation from the resting lung volume (ZEEP group) with physiologic tidal volumes caused histological damage of small airways, characterized by epithelial sloughing and rupture of alveolar-bronchiolar attachments (Table 1), with a concurrent increase in airway resistance and lung elastance that persisted after restoration of physiological end-expiratory volume (Fig. 2). In contrast with the observations in *ex vivo* rat models (5, 19, 25), these functional and morphologic alterations, which are closely comparable with those found in normal, open-chest rabbits subjected to the same mode of mechanical ventilation (7-9), occurred in the absence of inflammatory cytokine production, as indicated by the similarity of serum and BALF cytokine concentrations in the ZEEP and Control group (Fig. 4), and of parenchymal and pulmonary vascular injury (Table 1 and 2). Hence, damage of small airway epithelium and rupture of alveolar-bronchiolar attachments with tidal airway closure that occur in normal lungs during ventilation with physiological tidal volumes from resting lung volume do not induce the response known as *biotrauma* (10), in line with previous suggestions based only on measurements of BALF and serum TNF-α concentration in normal, closed- or open-chest rabbits ventilated for 3-4 hours at low lung volumes (8). In this connection, the possibility cannot be ruled out that longer periods of mechanical ventilation on ZEEP could eventually cause an inflammatory response characterized by the release of inflammatory cytokines: this response occurred, however, with the other types of injurious mechanical ventilation within the same time span as with ZEEP ventilation.

In normal rabbits, the histological damage of small airways and the permanent increase of airway resistance and lung elastance occurring with ventilation on ZEEP are due to increased surface tension, small airway collapse with gas trapping and microatelectasis, tidal airway closure and abnormally large stresses within the lungs (18). Indeed, administration of exogenous surfactant, by decreasing critical airway opening pressure, shear stress on small airway epithelium, and high stress in the alveolar-bronchiolar attachments, largely prevented both the histologic and functional alterations of mechanical ventilation at low lung volumes (9). Application of NEEP or induction of surfactant dysfunction with DOSS administration were therefore performed with the aim of increasing tidal airway closure, and hence, small airway injury. Based on E_{low}/E_{base} values (Fig. 2), the extent of tidal airway closure should have been in fact greater in the NEEP and ZEEP-DOSS groups than in the ZEEP group. Indeed, both the histological (Table 1 and 2) and mechanical alterations (Fig. 2

and 3) were also greater, and multiple linear regression analysis shows that Rint was significantly correlated with indices of bronchiolar injury (Fig. 6), besides lung wet-to dry ratio. The nature of the histological alterations differed in part between the NEEP and ZEEP-DOSS group. According to E_{low}/E_{base} values (Fig. 2), the extent of tidal airway closure should have been similar in animals of the NEEP and ZEEP-DOSS group, and in fact the histological damage of small airways (Table 1) and the mechanical alterations occurring during ventilation at low lung volume (Fig. 2) were also similar. On the other hand, substantial parenchymal and pulmonary vascular damage occurred in all animals of the ZEEP-DOSS group, probably because of higher surface tension, with marked interstitial or alveolar edema, while these alterations were present in mild degree only in two animals of the NEEP group (Table 2). In this connection, it should be noted that the more negative airway pressure (-7 to –10 cm H_2O) applied to a few rats, not included in the study, rapidly caused marked, deadly lung edema. After restoration of PEEP and repeated recruitment maneuvers, airway collapse and tidal airway closure were eliminated in all groups, as shown by E_{low}/E_{base} values being similar to those on $PEEP_1$ (Fig. 2), but because of lung edema, fewer units were being ventilated in the ZEEP-DOSS than in the NEEP group, as indicated by the end-expiratory volume on $PEEP_2$ being reduced in the former group and normal in the latter group (Fig. 3). Development of lung edema in the ZEEP-DOSS group is further supported by the high values of the wet-to-dry ratio (Table 2), and reduced diffusing capacity, as shown by the significant fall in arterial oxygen pressure (Fig. 1). This should explain the significantly greater mechanical changes occurring on $PEEP_2$ in the ZEEP-DOSS than in the NEEP and ZEEP group (Fig. 2), with the additional contribution from higher surface tension in the ZEEP-DOSS group.

Further reduction of the end-expiratory volume with negative airway pressure or augmentation of surfactant dysfunction at low volume with DOSS administration increased small airway epithelial damage relative to ventilation on ZEEP (Table 1), and, in contrast with the ZEEP group, both the NEEP and the ZEEP-DOSS group exhibited significantly higher concentration, relative to that of the Control and ZEEP group, of inflammatory cytokines in BALF and serum (Fig. 4). This suggests that greater damage and shedding of small airway epithelia and more extensive lesion of bronchiolar-alveolar attachments with enhanced tidal airway closure in the NEEP and ZEEP-DOSS group can eventually induce *biotrauma*. The discrepant inflammatory response to ventilation at ZEEP observed in the present *in vivo* and previous *in vitro* studies (5, 25) could be, therefore, related to greater susceptibility of the *in vitro* rat lung to noxious stimuli and/or presence of reparative processes in the *in vivo* lung. In this connection, it is of interest that ventilation with markedly negative airway pressure eventually induced a cytokine response in isolated mouse lung (3). Small airway injury does not seem, however, to represent the main contributor to the

inflammatory reaction that occurred with ventilation at low lung volume in normal rat lungs *in vivo*. Indeed, the release of inflammatory cytokines was substantially greater in the ZEEP-DOSS than in the NEEP group, except for TNF-α levels which did not differ significantly (Fig.4), in spite of the fact that indices of small airway injury were similar (Table 1). On the other hand, small airway injury was markedly more pronounced in the ZEEP-DOSS than the Hi-V_T group (Table 1), while the concentrations of pro-inflammatory cytokines were not significantly different (Fig. 4). Furthermore, studies performed on isolated, nonperfused lungs (5, 25) have missed indicating airway epithelium as the main source of inflammatory cytokines, although airway and alveolar epithelial cells were recognized effectors of inflammation (17,23). It was, instead, hypothesized (25) that activated leukocytes were responsible for the observed cytokine release, on the basis of a significant number of leukocytes being retained even in lungs perfused with saline for several hours (22).

In spite of bicarbonate administration, arterial pH decreased substantially throughout the period of mechanical ventilation (Fig. 1), reflecting the development of metabolic acidosis. Studies on cultured cells have provided conflicting results concerning the effects of pH on cytokine production (16). In the five groups of animals, however, pH did not differ significantly on $PEEP_1$, nor did its decrease from $PEEP_1$ to $PEEP_2$. Hence, whatever the influence of pH might have been, it cannot be responsible for the differences in the release of inflammatory cytokines that occurred with the various ventilatory strategies (Fig. 4).

Cultured, lung epithelial and endothelial cells subjected to mechanical stress eventually produce inflammatory mediators (17, 20, 27). In the present animals, release of inflammatory mediators occurred both during ventilation at low (ZEEP-DOSS and NEEP group) and high lung volumes (Hi-V_T group). Common to mechanical ventilation with large tidal volumes from physiological end-expiratory lung volume and mechanical ventilation with physiological tidal volumes from low end-expiratory lung volume is surfactant depletion and alteration of surface forces (1, 2, 28, 29), leading to alveolar instability, small airway collapse with dependent gas trapping or atelectasis, tidal airway closure, and regional overdistension that depend on the ventilatory mode, and are exaggerated in the presence of artificially induced surfactant dysfunction (ZEEP-DOSS group). Hence, the uneven, abnormally high stresses that develop at the alveolar and vascular level should cause increased epithelial and endothelial permeability (11,13), microvascular stress failure (14), and eventually interstitial or alveolar edema. Abnormal shear stress on endothelial cells, capillary stress failure, and disruption of the alveolar-capillary membrane can lead to the exposure of adhesion molecules and/or release of chemotactic factors, causing recruitment and activation of granulocytes (4, 20, 27), which could in turn represent the main contributors to

cytokine production (10). In fact, a rough parallelism occurred between levels of MIP-2, a chemotactic factor, in BALF of all groups (Fig.4) and corresponding semi-quantitative evaluations of alveolar granulocytes (Table2), although the absence of their phenotypic characterization might render the relevance of this relation questionable. This sequence of events, which has been proposed to explain the mechanisms of ventilator-induced lung injury in acute respiratory distress syndrome (21) could have in fact occurred in the present open-chest rats, as indicated by the significant correlation between the levels of inflammatory cytokines in BALF of all animals and the corresponding lung wet-to-dry ratios, taken as an index of the mechanical deformations responsible for vascular damage and, hence, interstitial or alveolar edema (Fig. 7). In contrast, no correlation was present between levels of inflammatory cytokines in BALF and indices of small airway injury. The link between stress induced vascular injury and inflammatory response is further supported by the significant correlation between cytokine concentration in serum and BALF that was observed in the present animals (Fig. 5), because this type of connection implies, in fact, loss of compartmentalization.

In conclusion, the present study has shown that in normal rat lungs 1) the histologic damage of the small airways due to tidal airway closure during mechanical ventilation with physiologic tidal volumes from the resting lung volume does not cause an inflammatory response characterized by release of inflammatory cytokines; 2) more extensive small airway alterations with enhanced tidal airway closure due to negative airway pressure or surfactant dysfunction eventually result in cytokine release; and 3) the main cause of the pulmonary and systemic inflammatory reaction with injurious modes of mechanical ventilation should be represented by stress related damage of endothelial and alveolar epithelial cells leading to development of lung edema.

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LEGENDS

Fig. 1. Mean values of arterial P_{O_2}, P_{CO_2}, and pH in open-chest rats during mechanical ventilation on positive end-expiratory pressure at the beginning ($PEEP_1$) and end of the experiment ($PEEP_2$) or from a low end-expiratory lung volume (EELV) because of zero (ZEEP) or negative end-expiratory pressure (NEEP). Each of the five groups entailed seven rats. Animals ventilated with physiological tidal volumes (8 $ml \cdot kg^{-1}$) on PEEP only represent the Control group, on ZEEP with or without dioctylsodiumsulfosuccinate administration the ZEEP-DOSS and ZEEP groups, and on NEEP the NEEP group, while animals ventilated with large tidal volumes (26 $ml \cdot kg^{-1}$) on PEEP only represent the Hi-V_T group. Bars: SE. Values significantly different from corresponding ones on $PEEP_1$: *P<0.05; **P<0.01.

Fig. 2. Mean values of interrupter resistance (Rint), quasi-static elastance (Est), viscoelastic resistance (Rvisc) and time constant (τvisc), and ratio of quasi-static elastance at volume changes of 1.3 and 8 $ml \cdot kg^{-1}$ (E_{low}/E_{base}; see Figure 3) in the various groups of open-chest rats (indications as in Fig. 1) during mechanical ventilation on positive end-expiratory pressure at the beginning ($PEEP_1$) and end of the experiment ($PEEP_2$) or from a low end-expiratory lung volume (EELV). Bars: SE. Values significantly different from corresponding ones on $PEEP_1$: *P<0.05; **P<0.01.

Fig. 3. Average relationship between volume changes from the resting lung volume (ΔV) and quasi-static transpulmonary pressure obtained in the baseline tidal volume range (8 $ml \cdot kg^{-1}$) during mechanical ventilation on positive end-expiratory pressure at the beginning ($PEEP_1$) and end of the experiment ($PEEP_2$) or from a low end-expiratory lung volume (EELV) in the various groups of open-chest rats (indications as in Fig. 1).

Fig. 4. Cytokine levels (median) in serum and bronchoalveolar lavage fluid (BALF) assessed at the end of the final period of mechanical ventilation on positive end-expiratory pressure (PEEP2) in the various groups of open-chest rats (indications as in Fig. 1). Values significantly different from those of the Control group: *P<0.05; **P<0.01.

Fig. 5. Relationships between cytokine concentration in serum and bronchoalveolar lavage fluid (BALF) at the end of the experiment in the Control (closed circles), ZEEP (open circles), NEEP (open triangles), ZEEP-DOSS (closed triangles), and Hi-V_T group (closed squares). Numbers are slope ±SE.

Fig. 6. Relationships between interrupter resistance (Rint) measured at the end of the experiment ($PEEP_2$) and bronchiolar injury score in the various groups of rats (see key to symbols). Numbers are B (slope) coefficient ±SE of multiple linear regression with bronchiolar injury score, abnormal bronchiolar-alveolar attachments, and wet-to-dry ratio as independent variables.

Fig. 7. Relationships between lung wet-to-dry ratio and cytokine concentration in bronchoalveolar lavage fluid obtained in the various groups of rats (see key to symbols). Numbers are B (slope) coefficient ±SE of multiple linear regression with wet-to-dry ratio, bronchiolar injury score, and abnormal bronchiolar-alveolar attachments as independent variables.

Table 1. Indices of bronchiolar injury in rats after 2-2½ hours of mechanical ventilation with various ventilatory strategies

	N	IS (%)	A-A (%)	D (μ)
Control group	7	6.8 (6-9)	8.9 (5-10)	52 (47-59)
ZEEP group	7	30.9* (27-33)	31.1* (23-40)	73* (64-86)
NEEP group	7	42.9* (33-52)	38.8* (29-43)	83* (68-89)
ZEEP-DOSS group	7	42.4* (32-57)	34.5* (24-41)	77* (58-88)
Hi-V_T group	7	9.0 (8-14)	19.7^{+} (15-23)	62 (47-68)

Values are medians with range in parentheses. N=number of animals; IS, bronchiolar injury score; A-A, percentage of ruptured bronchiolar-alveolar attachments; D, distance between normal bronchiolar-alveolar attachments. Ventilation with physiological tidal volume (8 ml/kg) on positive end-expiratory pressure only (Control group), zero end-expiratory pressure (ZEEP group), negative end-expiratory pressure (NEEP group), or zero end-expiratory pressure after dioctylsodium-sulfosuccinate administration (ZEEP-DOSS group), and ventilation with large tidal volume (26 ml/kg) on positive end-expiratory pressure only (Hi-V_T group). Significantly different from Control group: $^{+}P<0.05$; *$P<0.01$.

Table 2. Indices of parenchymal and vascular injury after 2-2½ hours of mechanical ventilation with various ventilatory strategies

	Parameters	Injury score/ Number of rats				
		0	1	2	3	
Control group	focal alveolar collapse	7				
	edema	7				
	hemorrhage	7				
	alveolar granulocytes	7				
	W/D					4.25±0.07
	ABALF/ASER %					1.4±0.1
ZEEP group	focal alveolar collapse	7				
	edema	7				
	hemorrhage	7				
	alveolar granulocytes	6	1			
	W/D					4.29±0.06
	ABALF/ASER %					1.9±0.3
NEEP group	focal alveolar collapse	6	1			
	edema	4	1	2		
	hemorrhage	7				
	alveolar granulocytes	5	2			
	W/D					4.73±0.13
	ABALF/ASER %					3.4±0.5
ZEEP-DOSS	focal alveolar collapse	5	2			
	edema	0	1	3	3	
	hemorrhage	5	2			
	alveolar granulocytes	2	2	2	1	
	W/D					7.28±0.10*
	ABALF/ASER %					21.3±0.9*
Hi-VT group	focal alveolar collapse	6	1			
	edema	2	3	2		
	hemorrhage	6	1			
	alveolar granulocytes	3	2	2		
	W/D					5.34±0.09*
	ABALF/ASER %					7.6±0.7*

Ventilation with physiological tidal volume (8 ml/kg) on positive end-expiratory pressure only (Control group), zero end-expiratory pressure (ZEEP group), negative end-expiratory pressure (NEEP group), or zero end-expiratory pressure after dioctylsodium-sulfosuccinate administration (ZEEP-DOSS group), and ventilation with large tidal volume (26 ml/kg) on positive end-expiratory pressure only (Hi-VT group). Lung wet-to dry ratio (W/D) and percent ratio of BALF to serum albumin concentration (ABALF/ASER %). Injury score: 0=absent, 1=mild, 2=moderate, 3=marked. A rough evaluation of group average alveolar granulocytes is obtained as the sum of number of rats time corresponding injury score in each group divided by the maximal possible sum, i.e. total number of rats time maximal injury score. Significantly different from Control group: $^{*}P<0.05$

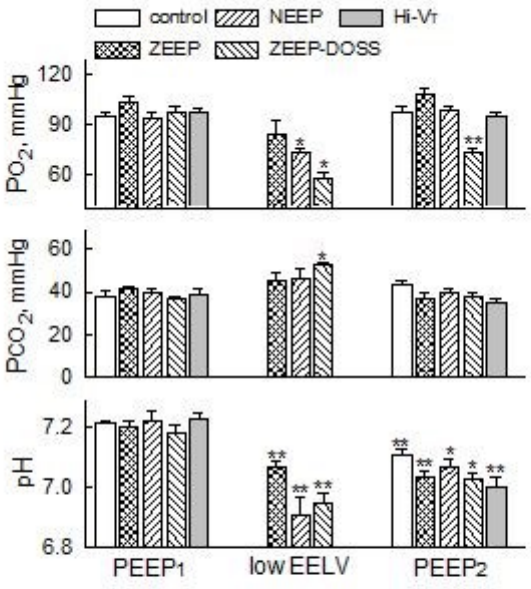
control
NEEP
Hi-VT
ZEEP
ZEEP-DOSS
PO2, mmHg
120
90
60
PCO2, mmHg
60
40
20
0
pH
7.2
7.0
6.8
PEEP1
low EELV
PEEP2

Figure 1

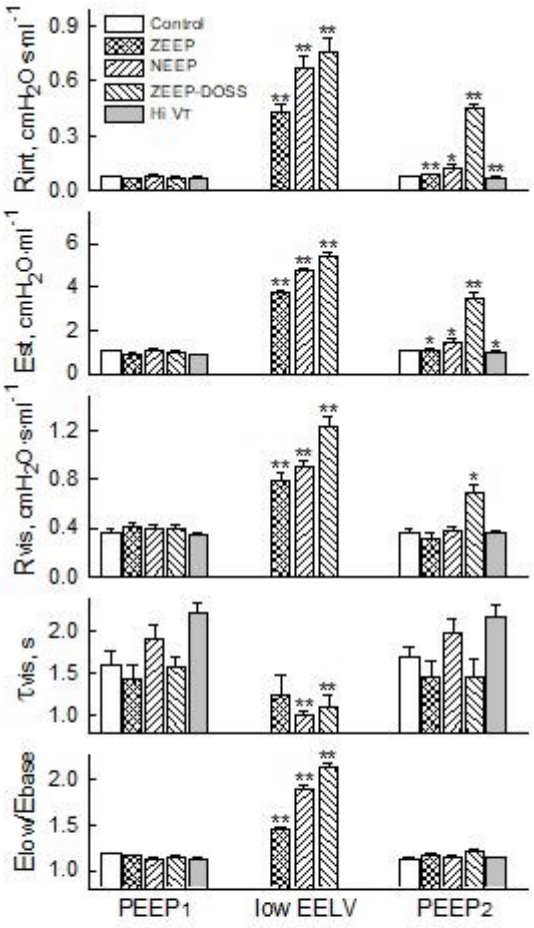
Control
ZEEP
NEEP
ZEEP-DOSS
Hi VT
Rint, cmH2O·s·ml-1
Est, cmH2O·ml-1
Rvis, cmH2O·s·ml-1
τvis, s
Elow/Ebase
PEEP1
low EELV
PEEP2

Figure 2

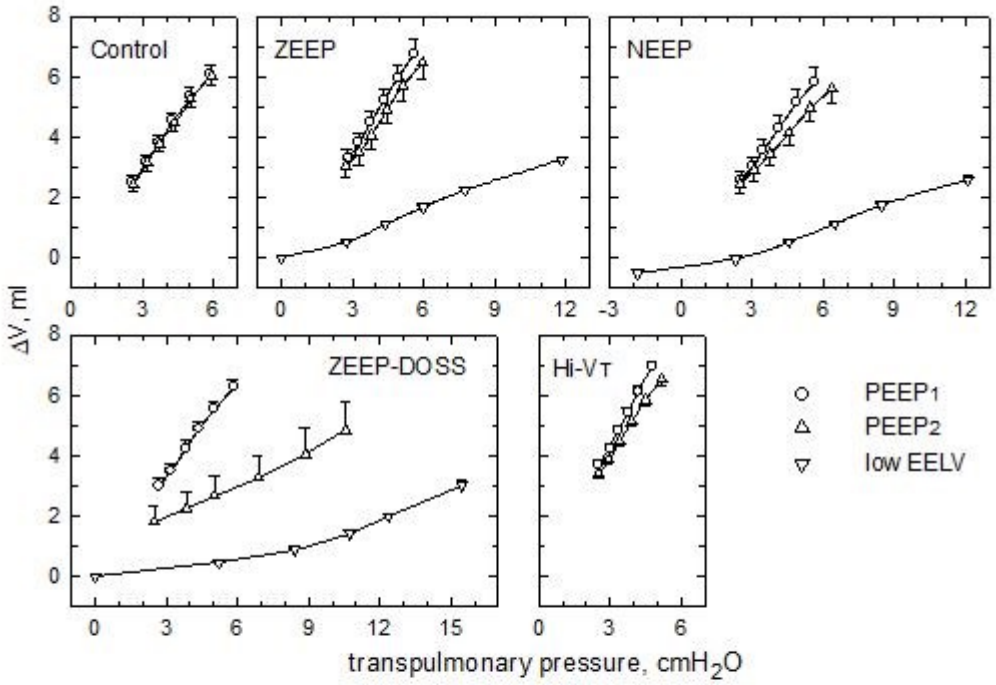
Control
ZEEP
NEEP
ZEEP-DOSS
Hi-VT
PEEP1
PEEP2
low EELV
ΔV, ml
transpulmonary pressure, cmH2O

Figure 3

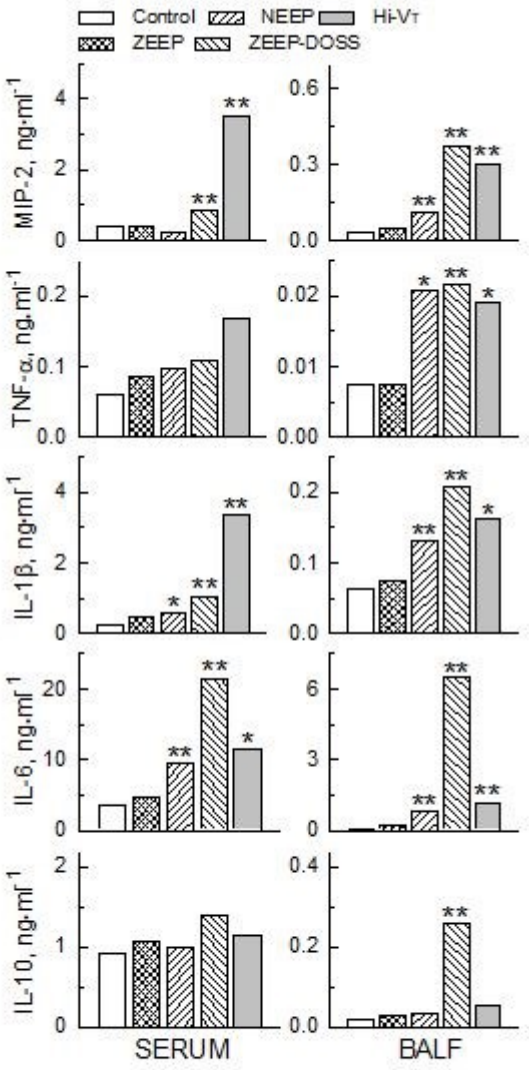
Control
NEEP
Hi-VT
ZEEP
ZEEP-DOSS
MIP-2, ng·ml-1
TNF-α, ng·ml-1
IL-1β, ng·ml-1
IL-6, ng·ml-1
IL-10, ng·ml-1
SERUM
BALF

Figure 4

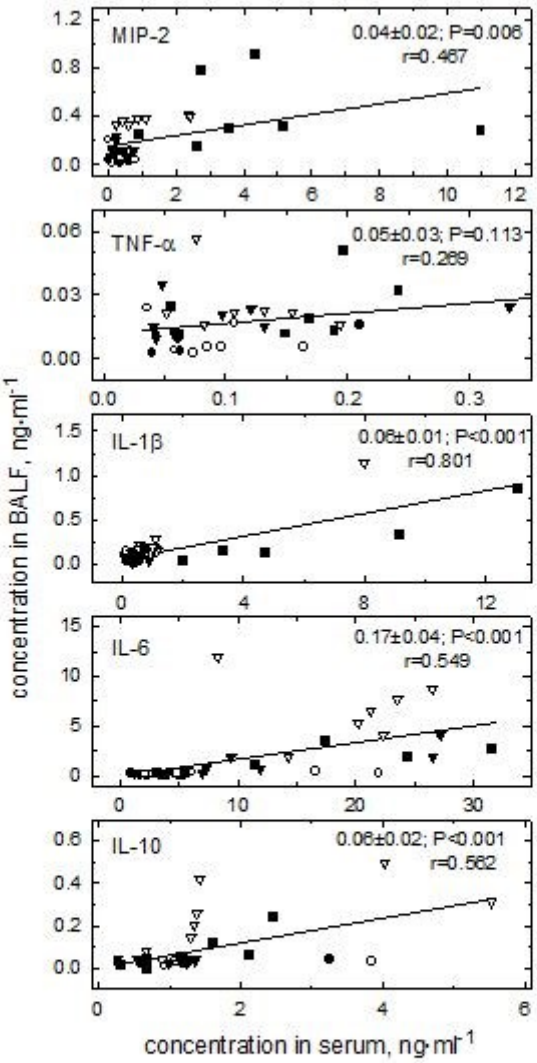
MIP-2
0.04±0.02; P=0.006
r=0.467
TNF-α
0.05±0.03; P=0.113
r=0.269
IL-1β
0.06±0.01; P<0.001
r=0.801
IL-6
0.17±0.04; P<0.001
r=0.549
IL-10
0.06±0.02; P<0.001
r=0.562
concentration in BALF, ng·ml-1
concentration in serum, ng·ml-1

Figure 5

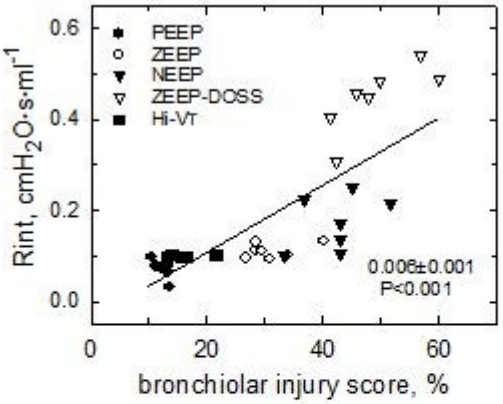
PEEP
ZEEP
NEEP
ZEEP-DOSS
Hi-Vt
0.006±0.001
P<0.001
Rint, cmH2O·s·ml-1
bronchiolar injury score, %
0.0
0.2
0.4
0.6
0
20
40
60

Figure 6

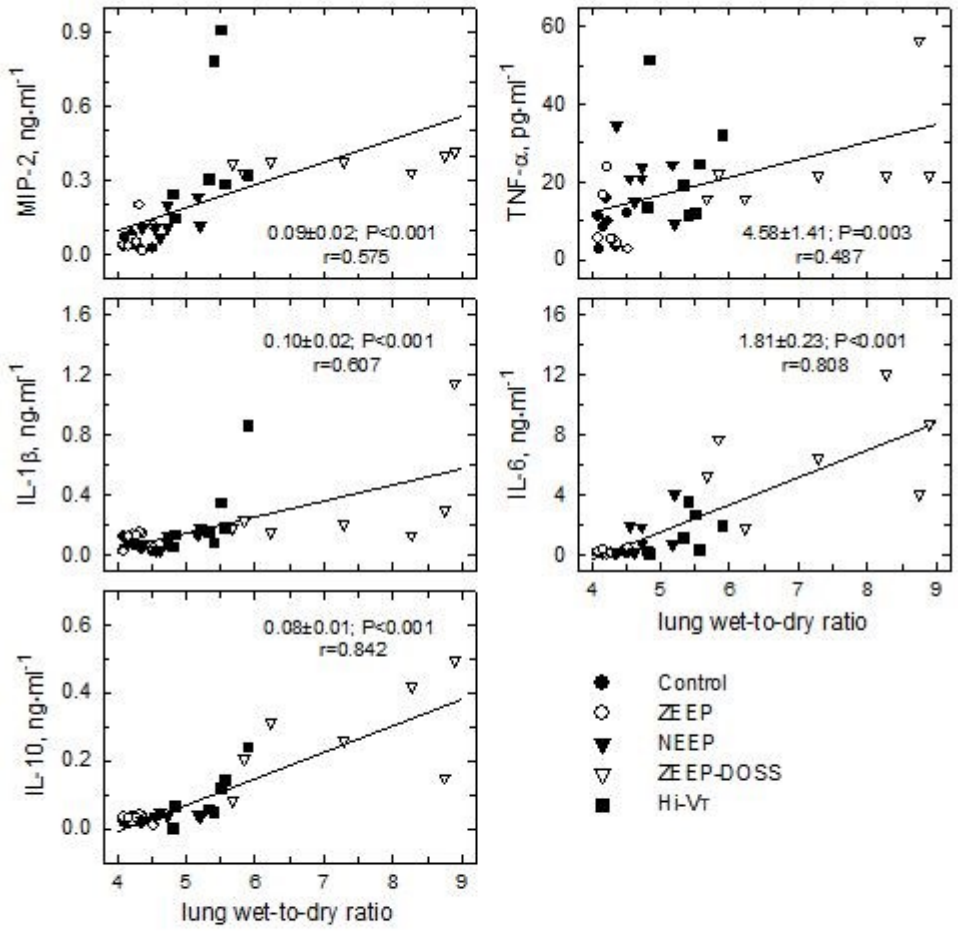
MIP-2, ng·ml-1
0.09±0.02; P<0.001
r=0.575
TNF-α, pg·ml-1
4.58±1.41; P=0.003
r=0.487
IL-1β, ng·ml-1
0.10±0.02; P<0.001
r=0.607
IL-6, ng·ml-1
1.81±0.23; P<0.001
r=0.808
IL-10, ng·ml-1
0.08±0.01; P<0.001
r=0.842
lung wet-to-dry ratio
lung wet-to-dry ratio
Control
ZEEP
NEEP
ZEEP-DOSS
Hi-VT

Figure 7