The development of various forms of lung injury with increasing tidal volume in normal rats.

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Abstract

Sixty-three, open-chest normal rats were subjected to mechanical ventilation (MV) with tidal volumes (V_T) ranging from 7.5 to 39.5ml·kg^{-1} and PEEP 2.3cmH_2O. Arterial blood gasses and pressure, and lung mechanics were measured during baseline ventilation (V_T=7.5ml·kg^{-1}) before and after test ventilation, when cytokine, von Willebrand factor (vWF), and albumin concentration in serum and broncho-alveolar lavage fluid (BALF), wet-to-dry weight ratio (W/D), and histologic injury scores were assessed. Elevation of W/D and serum vWF and cytokine concentration occurred with V_T>25ml·kg^{-1}. With V_T>30ml·kg^{-1} cytokine and albumin concentration increased also in BALF, arterial oxygen tension decreased, lung mechanics and histology deteriorated, while W/D and vWF and cytokine concentration increased further. Hence, the initial manifestation of injurious MV consists of damage of extra-alveolar vessels leading to interstitial edema, as shown by elevated vWF and cytokine levels in serum but not in BALF. Failure of the endothelial-epithelial barrier occurs at higher stress-strain levels, with alveolar edema, small airway injury, and mechanical alterations.

Key words: Cytokines; Lung edema; Lung mechanics; Lung histology; Mechanical ventilation; von Willebrand factor

1. Introduction

Although in many instances mechanical ventilation (MV) is an essential therapeutic intervention, several animal studies have demonstrated that it can result in severe lung injury or enhance the pre-existing lung damage (Webb and Tierney, 1974; Egan 1982; Parker et al., 1984; Dreyfuss et al., 1988). The functional and histologic alterations induced by mechanical ventilation (MV) in experimental animal models do not substantially differ from those occurring in human acute respiratory distress syndrome, and this enhances the interest in the results obtained with injurious MV in normal animals (Dreyfuss and Saumon, 1998). Indeed, the pathophysiologic and clinical relevance of the observations in animal models, especially in normal animals in which the noxious effects cannot be related to pre-existing alterations, has been proven by the ARDS Network study (2000).

The prevailing opinion about the cause of the deleterious effects of ventilator induced lung injury (VILI) is that excessive tissue strain eventually increases endothelial permeability and hence causes plasma extravasation (Dreyfuss and Saumon, 1998). In contrast, it has been proposed that the initial damage consists of surfactant depletion due to prolonged use of sufficiently large tidal volumes, with increased surface tension, consequently enhanced Starling filtration forces, and eventually alteration of epithelial permeability (Maruscak et al., 2008). Inherent to all these views is the concept of the presence of a threshold for the production of adverse effects, usually quantified by the tidal volume (V_T) or transpulmonary pressure changes. However, marked differences have been reported among species in the sensitivity to the noxious action of MV and the dependent development of VILI (Dreyfuss and Saumon, 1998), and this casts doubts on the precise level or even the presence of such threshold. Indeed, it has been suggested that MV is always injurious (Moriondo et al., 2007; Vaneker et al. 2007; Wolthuis et al., 2009), only the amount of damage being related to the level of tissue strain (Moriondo et al., 2007 Wolthuis et al., 2009).

The dependence of edema production on the magnitude of the V_T used to ventilate normal lungs from the physiological end-expiratory volume has been documented with some accuracy in dogs and pigs (Parker et al., 1990; Protti et al., 2011), whereas that of the mechanical alterations, histologic lesions of lung parenchyma and peripheral airways, and release of inflammatory markers has been evaluated only approximately. This is especially the case of the inflammatory response, when only three V_T levels have been generally used (Webb and Tierney, 1974; Muscedere et al., 1994; Tremblay et al., 1997). In addition, although the connection between mechanical stress produced by MV and cellular processes capable to trigger an inflammatory reaction in the lung has been documented (Tremblay and Slusky, 1998), no lung or systemic inflammatory reaction to noxious MV in normal animals has been observed in some studies (Dreyfuss and Saumon, 1998; Verbrugge et al., 1999; Dreyfuss et al., 2003). Furthermore, no information exists concerning the release of

markers of vascular damage and its dependency on the strain of lung tissue during MV in vivo. Finally, no previous study has simultaneously assessed the V_T dependence of all these adverse effects in normal animals. Here, we have investigated the development of the various forms of lung injury that occur in normal, open-chest rats during MV with several levels of V_T.

2. Methods

Sixty-three male Sprague-Dawley rats (weight range 370-450 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 home-made ventilator (D'Angelo et al. 2002). Anesthesia and complete muscle relaxation were maintained with additional doses of the anesthetic mixture and pancuronium bromide. Adequacy of anesthesia was judged from the absence of sudden increases 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 an appropriate positive end-expiratory pressure (PEEP; 2.3±0.01 cmH_2O) maintained the end-expiratory lung volume of the intact condition.

Airflow 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 (Pa) 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 (D'Angelo et al., 2002), delivering water-saturated air at constant flow of selected magnitude 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 or to a drum in which the pressure was adjusted by means of a flow-through system. During measurements of lung mechanics, the ribs and diaphragm were pulled widely apart, in order to prevent contact between lung and chest wall, except in their dependent parts.

2.1 Procedure and data analysis

Figure 1 provides a time line representation of the procedures performed in the seven groups of animals. All rats underwent an initial and final 15 min period of baseline ventilation (control and test condition, respectively) during which arterial blood gasses, pH, and lung mechanics were assessed. Control and test condition were separated by a 2 or 4 hrs period during which tidal volume (V_T) was either kept at baseline value or set to one of the six selected values (test period). The animals were randomly assigned to one of the seven V_T groups. Table 1 reports the ventilatory variables for the 7 groups of rats.

Assessment of lung mechanics was made using the rapid end-inflation occlusion method (D'Angelo et al., 2002). While keeping V_T at baseline values, 21 test inflations of 7 different durations in the range 0.25 to 3 s were intermittently performed, and end-inspiratory occlusions lasting 5 s were made in all test breaths. The lungs were inflated 3-4 times to a transpulmonary pressure (Ptp) 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 ($\Delta EELV$), the latter being the volume at zero Ptp. Quasi-static elastance (Est), interrupter (airway) resistance (Rint), and viscoelastic resistance (Rvisc) and time constant ($\tau visc$) at baseline V_T were computed as previously reported (D'Angelo et al., 2002).

After completion of the mechanics measurements, 1.5–2 ml of blood were drawn from the heart for the assessment of cytokine and 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 $ml \cdot kg^{-1}$ of normal saline in two aliquots, fluid recovery ranging from 40 to 60%, 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).

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

2.2 Cytokine and albumin assessments

Cytokine (TNF-α, IL-1β, 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 $pg \cdot ml^{-1}$, respectively, in which case concentration was assumed to be nil. Sandwich enzyme immunoassay of E-Selectin and von Willebrand Factor (vWF) was performed using goat anti-rat E- Selectin antibodies (R&D Systems Inc, MN, USA) and sheep anti-rat vWF antibodies (Affinity Biologicals Inc, Ancaster, Ontario, Canada). Absorbance was read at 405 nm. A pool of sera from 20 healthy rats was used as the standard control, and readings from individual specimens were expressed as percent of control.

The albumin concentration of BALF supernatant and in 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.

2.3 Histologic examination

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 (D'Angelo et al., 2002, 2004, 2007). The following measures were obtained using a computer-aided, image analysis system (IMAQ Vision for LabView; National Instruments, Austin, TX): a) the mean linear intercept (Lm), a measure of air-space enlargement, and its coefficient of variation as a measure of dispersion; *b)* the percent ratio of abnormal to total (normal plus abnormal) bronchiolar-alveolar attachments, and the distance between normal attachments, as an indices of airway-parenchymal mechanical uncoupling; and *c)* the percent ratio of injured (epithelial necrosis and sloughing) to total

membranous bronchioles, the bronchiolar injury score (IS), as an index of small airway damage (Muscedere et al., 1994).

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

2.4 Statistics

Analyses were performed using SPSS 25 (SPSS Inc., Chicago, IL). Results from mechanical studies, measurements of arterial blood gasses and pH, and alveolar dimensions are presented as means±SE. Comparisons among conditions were performed using ANOVA and Bonferroni post-hoc test. Results from marker assessments and histological studies are expressed as median and range, statistical analysis being performed with the Kruskal-Wallis test and Bonferroni post-hoc test. Regression analysis was performed using the least square method. A two-tailed $P \leq 0.05$ was considered statistically significant.

3. Results

Under control condition, no significant differences occurred among groups in any of the measured variables (Fig. 2 and 6). All animals of group 1 to 5 completed the scheduled period of test ventilation without the need of supportive interventions. In contrast, the 2 hrs period was shortened by ~10 min in 2 rats of group 6, and by 15 to 30 min in 4 rats of group 7, as signs of instability became manifest. Furthermore, some rats of these groups had to be aspirated because of foam in the trachea.

On resumption of baseline ventilation (test condition), pHa was significantly decreased by nearly the same amount in all groups, Pa_{O_2} was decreased in group 6 and 7, and significantly more in the latter group, whereas Pa_{CO_2} and Pa did not differ significantly from control values in all groups (Fig. 2).

3.1 Edema formation

The mean values of W/D and BALF to serum albumin concentration ratio (A_{BALF}/A_{SER}) were similar in groups 1 to 4 (Fig. 3); their cumulative averages (4.38±0.04 and 1.7±0.1%) were not significantly different from those obtained in 25 normal rats instrumented as described above and immediately killed with an overdose of anesthetic (D'Angelo et al., 2008; Loring et al., 2010; Pecchiari et al., 2014). In contrast, W/D increased progressively from group 5 to 7, whereas

ABALF/ASER increased above control values in group 6 and 7 only, and significantly more in the latter group (Fig. 3). Inspection of individual data obtained in animals subjected to test ventilation for 2 hrs (group 1-3 and 5-7) showed that a peak inflation pressure (Ppeak) >25 cmH_2O, was required to significantly increase W/D above the normal range (mean+3 SD), and a W/D >5.8 to significantly increase ABALF/ASER above normal values, i.e to cause plasma extravasation into the airspaces (Fig. 3). Increasing the duration of test ventilation to 4 hrs and keeping Ppeak <25 cmH_2O. as in group 4 (Table 1), did not change the threshold levels, because in this group the mean values of both W/D and ABALF/ASER were still normal.

3.2 Cytokines release

Fig. 4 shows the levels concentration of inflammatory and anti-inflammatory cytokines in serum and BALF for the various groups of rat. In serum, the levels of IL-1, IL-6, MIP-2, and IL-10 were similar in groups 1 to 4, and were significantly increased to similar levels in groups 5 to 7, except for MIP-2 which exhibited a further significant increase from group 5 to 6. In contrast, TNF-α did not differ significantly among groups. In BALF, the cytokine concentration did not differ among groups 1 to 5, and increased to similar levels in groups 6 and 7.

Serum levels of vWF were normal in group 1 to 4, but increased progressively from group 5 to 7 (Fig. 5). In contrast, the late endothelial factor E-selectin, though increased in the last groups, never differed significantly from normal.

3.3 Histology

Scores of airway-parenchymal uncoupling (% abnormal bronchiolar-alveolar attachments, distance between normal attachments), and bronchiolar epithelial injury (IS) are shown in Table 2, while those of parenchymal and vascular injury are summarized in Table 3. Except for marginal amount of focal alveolar collapse in group 3-4, no histologic evidence of parenchymal and vascular damage, airway-parenchymal uncoupling, and bronchiolar epithelial injury was found in group 1 to 4. Substantial evidence of perivascular edema was present in group 5. In contrast, group 6 and 7 exhibited marked signs of perivascular edema, bronchiolar epithelial injury (IS), airway-parenchymal uncoupling, and alveolar infiltration of granulocytes, while peribronchial edema, hemorrhage, and reduction of airspace dimensions with increased anisotropy, evaluated from the coefficient of variation of Lm, occurred more consistently in group 7.

3.4 Lung mechanics

Under test condition, all parameters remained unchanged in groups 1 to 5 (Fig. 6). In contrast, Est, Rint, and Rvisc increased and ΔEELV decreased in group 6 and 7, and significantly more in the latter group. In spite of a tendency to decrease in group 6, the fall of the viscoelastic time constant was significant only in group 7.

Satisfactory relationships were found between the increase of W/D above normal values and the concomitant changes of Est or Rint (Fig. 7). A significant relationship occurred also between the changes of Rint and the increase of ruptured broncho-alveolar attachments above group 1 to 5 median.

4. Discussion

This is the first study assessing the dependence of all the various aspects of lung injury at progressively increasing tidal volume. The initial manifestation of injurious MV consists of vascular stress-strain failure leading to perivascular edema, increased W/D, and elevated vWF and cytokine levels in serum, with no involvement of the alveolar and airway compartments. This occurs once the tidal volume and/or the peak transpulmonary pressure exceed a critical threshold, which in the present rats should have occurred with VT and Ppeak of 26-27 $ml \cdot kg^{-1}$ and 26-27 cmH_2O, respectively. With VT and Ppeak of 35 $ml \cdot kg^{-1}$ and 40 cmH_2O or greater, the alveolar and peripheral airway compartments are eventually involved, as shown by histological damage, alveolar and peribronchial edema, increased cytokine levels in serum and BALF and albumin concentration in BALF, fall of arterial oxygen tension, and deterioration of lung mechanics. This indicates that there is the potential for different mechanisms leading to perivascular or alveolar edema, with the former preceding the onset of the latter.

4.1 Edema formation and cytokine release

No morpho-functional alterations were observed in animals ventilated with VT up to 22.3 $ml \cdot kg^{-1}$ for 2 hrs nor in animals ventilated for 4 hrs with an even higher VT of 24.3 $ml \cdot kg^{-1}$ (Fig. 2-6; Tables 2 and 3), indicating that no noxious time-dependent effects occur within this time span in groups 1 to 4. Some functional alterations appeared only within 2 hrs of ventilation with VT and Ppeak of 29 $ml \cdot kg^{-1}$ and 29.5 cmH_2O, respectively (group 5). This is in accordance with previous studies which showed that mechanical ventilation with VT and peak inspiratory pressures up to 20 $ml \cdot kg^{-1}$ and 18 cmH_2O, respectively, is not injurious to normal rat lungs (Webb and Tiernney, 1974, Tremblay et al., 1997; Allen et al JAP 2005), and that the capillary filtration coefficient and lung wet-to-dry weight ratio remain unchanged during ventilation with Ppeak of 25 cmH_2O in an ex vivo rabbit

preparation (Hernandez et al,, 1990). While VILI is a threshold phenomenon (Carlton et al., 1990), the search for a univocal threshold is likely unwarranted even limited to normal lungs and single species, given the many factors involved. The present observations suggest that V_T and Ppeak >25 $ml \cdot kg^{-1}$ and >25 cmH_2O are required to initiate lung edema in healthy, open-chest rats (Fig. 3). However, mild signs of interstitial edema, moderate histological damage and inflammatory reaction, but no mechanical alterations and impairment of gas exchange, have been found in rats ventilated with V_T of 25-26 $ml \cdot kg^{-1}$ (Dreyfuss et al., 1995; D'Angelo et al., 2008). Altogether, these observations support the conclusion that no VILI occurs during MV with ~3.5 times the eupneic V_T observed in spontaneously breathing, anesthetized rats (D'Angelo et al., 2008; Loring et al., 2010; Pecchiari et al., 2014).

MV with V_T of 29 $ml \cdot kg^{-1}$ (group 5) resulted in perivascular edema and increased W/D, which are adequately explained by the concomitant elevation of vWF (Table 3; Fig. 3 and 5), a factor overexpressed by injured endothelial cells and exposed subendothelial connective tissue (Sadler, 1998; Müller et al., 2002). There was also a modest increase of cytokine concentration in serum (Fig. 4), which can results from excessive cyclic stretch with endothelial cell disruptions, and direct contact between polymorphonuclear cells and basement membrane causing their activation (Dreyfuss and Saumon, 1994). Furthermore, abnormal stress-strain of endothelial cells can also induce upregulation of genes responsible for cytokine synthesis (Grembowicz et al., 1999), or stimulate the formation of microparticles shed from the surface of injured endothelial cells, acting as inflammatory stimuli (Morel et al., 2009; Letsiou et al., 2013). These mechanisms were probably acting in group 6 and 7, when the inflammatory reaction was substantially enhanced (Fig. 4).

In group 5, alveolar and peripheral airway compartments were not involved; lung mechanics (Fig. 6), gas exchange (Fig. 2), indexes of morphological alterations (Table 3), BALF-to-serum albumin concentration ratio (A_{BALF}/A_{SER}) and cytokine levels in BALF (Fig. 4 and 5) were in fact unaffected. Development of perivascular edema without apparent alveolar edema has been previously seen in intact rats mechanically ventilated from the resting lung volume with peak inflation pressure of 30 cmH_2O for about one hour (Webb and Tierney, 1974). These observations are consistent with the effects of lung interdependence (Howell et al., 1961; Benjamin et al., 1974), which does not affect the alveolar capillaries, but should decrease the pressure in the space surrounding the extra-alveolar vessels, thus allowing fluid accumulation, also in combination with endothelial damage, testified by the increase of vWF. While this supports the presence of different mechanisms for perivascular and alveolar edema formation, it cannot be excluded that in group 5 animals, the cyclic, substantial strain of the alveolar capillaries had increased their permeability thus producing interstitial edema, or that this would have occurred if MV had been prolonged beyond the

scheduled 2 hrs. The thickness of alveolar septa was not apparently increased in the histologic specimens from this group, but this could depend on the relatively high inflation pressure applied during the fixation procedure, besides the limitation of optical microscopy.

Cytokines levels in serum were definitely increased in groups 5 to 7 (Fig. 4), and E-selectin is known to be transcriptionally activated in response to cytokines. Neverthelesss, serum levels of E-selectin, though somewhat higher in groups 6 and 7, were not significantly different from normal (Fig. 5). This probably relates to the fact that in response to IL-1 or TNF-α injection, serum levels of E-selectin might become elevated only after several hours (Bevilacqua et al., 1989; Pilewski et al., 1994).

4.2 Alveolar edema and airway involvement

Using larger VT and Ppeak (group 6 and 7) resulted in marked morpho-functional alterations, which were more pronounced in the latter group. The picture was dominated by extensive alveolar edema and fluid accumulation in the airways, with elevated cytokine concentrations and abnormally high ABALF/ASER, besides hemorrhages and infiltration of leukocytes into the alveoli (Table 3 and Fig, 3). Interestingly, there was a tight relationship between W/D and ABALF/ASER above control value, an index of fluid extravasation into the alveoli; this suggests that a critical level in terms of W/D should exist for alveolar edema formation, which in the present animals was ~5.8 (Fig. 3). Inflation to high lung volumes is known to cause stress failure of pulmonary capillaries, with increased permeability and disruptions of the endothelial layer, most likely because of enhanced longitudinal forces (Egan 1982; Parker et al., 1984, Fu et al., 1992; West and Mathieu-Costello, 1999). Furthermore, because of the high stresses exerted at large lung volumes on the extra-alveolar structures due to interdependence phenomena, especially in the presence of heterogeneous expansion (Howell et al., 1961), peribronchial edema developed and perivascular edema, firstly seen in group 5, increased further (Table 3).

Although the study was not designed to determine the factors involved in the rate at which lung edema develops, it was apparent that the time course of edema formation differed among group 6 and 7 rats. In 4 animals of group 7, extensive alveolar edema, with fluid accumulation in the airways (Fig. 3), marked fall of blood oxygenation because of reduced lung diffusing capacity and increased venous admixture (Fig. 2), and emerging cardiovascular problems, obliged to stop test ventilation 10 to 30 min before the scheduled 2 hours, and to prematurely return to baseline ventilation for the completion of mechanical measures. In contrast, this happened in only two rats of group 6, and for a premature return to baseline ventilation of only 10 min.

4.3 Mechanical properties

Lung mechanical variables did not differ significantly among groups 1 to 4 (Fig. 6), i.e. when W/D, ABALF/ASER, cytokine levels, and histology were similar among groups (Table 2 and 3) and within normal limits. No change in mechanical properties occurred in group 5, in spite of increased W/D, cytokine levels in serum, and substantial histologic signs of perivascular edema (Table 2 and 3). It seems therefore that fluid accumulation in the extra-alveolar space has no effects on lung mechanical properties, at least for increases in W/D like those occurring in group 5. Conversely, monitoring lung mechanics represents an inadequate mean for the evaluation of developing pulmonary edema.

Significant alterations of lung mechanics, consisting of increased static elastance, airway and viscoelastic resistance, and decreased viscoelastic time constant (Fig. 6), occurred only in group 6 and 7, i.e. only once alveolar edema had developed. Changes of Est and Rint were mainly due to alveolar edema and fluid accumulation in the airways, as suggested by the good relationship between the changes of these parameters and the concomitant increases of W/D (Fig. 7). Reduction of ventilated parenchyma, as shown by the fall of the end-expiratory lung volume (Fig. 6) and the presence of widespread focal alveolar collapse (Table 3), explains the increase of Est and Rint, besides Rvisc. Enhanced alveolar tidal stretch and/or plasma extravasation into the air spaces (Wyszogrodski et al. 1975; Da Silva et al. 2005; Maruscak et al., 2008) have been shown to increase surface tension, thus providing an additional cause of increased parenchymal stiffness and airway collapse. Indeed, only in group 6 and 7 there was an increase of ABALF/ASER (Fig. 3), and a decrease of Lm at fixed transpulmonary pressure (Table 2).

Parenchymal and small airway involvement was markedly heterogeneous as indicated by substantially increased dispersion of alveolar dimensions (Table 2), and by atelectatic foci, peribronchial edema, and bronchiolar damages (Table 3) being highly scattered throughout the lung. The reduction of viscoelastic time constants (Fig. 6) also support the presence of heterogeneous mechanical properties; estimated τvisc and Rvisc are in fact predicted to decrease and to increase, respectively, by the analysis of a viscoelastic lung model (D'Angelo et al. 2019), implemented with several, equal parallel units, when only Rint is made to increase markedly in a consistent number of those units. Heterogeneity of lung expansion during tidal ventilation with large VT's should have produced uneven, abnormally high stresses, which should have further enhanced epithelial and endothelial permeability (Egan 1982; Dreyfuss et al., 1988; Fu et al., 1992) and produced bronchiolar injury, possibly via cyclic small airway opening and closing, causing bronchiolar-alveolar uncoupling that contributed to the increase of airway resistance (Fig. 7). Interestingly, mechanical and histological alterations and inflammatory reactions observed in normal rats

mechanically ventilated with the highest VT's and physiological end-expiratory transpulmonary pressure (group 6 and 7; Table 2 and 3, and Fig. 6) were qualitatively similar to those observed in normal rats mechanically ventilated with protective VT and markedly lowered EELV with or without surfactant depletion (D'Angelo et al., 2008). Common to the two experimental conditions is in fact the development of markedly uneven stresses and strains throughout the lung.

4.4 Concluding remarks

This study, in which the evolution of the various forms of lung damage at progressively increasing tidal volume has been investigated for the first time, shows that in normal rats lung injury occurs once a definite VT threshold is overcome. Stress-strain failure of extra-alveolar vessels leading to interstitial edema is the initial manifestation of injurious MV, as evidenced by elevated vWF and cytokine levels in serum but not in BALF. Failure of the endothelial-epithelial barrier with alveolar flooding occurs at substantially higher stress-strain levels, with substantial inflammatory reaction, mechanical alterations and small airway damage proportional to the concomitant edema formation. In the present animals, the threshold for the formation of extra-alveolar interstitial edema was ~3.7 times the eupneic VT of spontaneously breathing, anesthetized rats, and ~4.5 times the eupneic VT for the formation of alveolar edema.

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Table 1. Ventilatory variables used in normal open-chest rats during the test period of mechanical ventilation

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
N	9	9	9	9	9	9	9
b.w., kg	0.42±0.01	0.42±0.01	0.42±0.01	0.44±0.01	0.43±0.01	0.42±0.01	0.43±0.01
TP, hrs	2	2	2	4	2	2	2
V_T, ml·kg^{-1}	7.5±0.02	15.3±0.3	22.3±0.5	24.3±0.4	29.1±0.5	37.1±0.6	39.5±0.8
Ppeak, cmH_2O	6.1±0.1	11.6±0.3	18.4±0.5	23.8±0.3	29.5±0.5	40.4±0.8	49.3±0.4
T_I, s	0.45	0.78±0.01	1.15±0.07	1.16±0.02	1.48±0.07	1.75±0.03	1.88±0.05
T_E, s	0.50	1.20±0.04	1.71±0.16	2.05±0.03	2.68±0.16	3.01±0.11	3.18±0.11
$\dot{V}_I$,ml·kg^{-1}·min^{-1}	475±1	465±14	473±10	454±8	423±10	469±7	470±6

Values are means±SE. N, number of animals; b.w., body weight; TP, duration of the test period; V_T, tidal volume; Ppeak, peak transpulmonary pressure; T_I, duration of inflation; T_E, expiratory duration; $\dot{V}_I$, pulmonary ventilation.

Table 2. Indices of airspace expansion and bronchiolar injury in normal open-chest rats mechanically ventilated with different tidal volumes

	Lm μ	CV of Lm %	IS %	A-A %	D_{A-A} μ
Group 1	71±5	27±4	8.7 (8-14)	11.0 (10-17)	43 (37-60)
Group 2	72±5	28±3	9.4 (6-16)	12.3 (9-18)	49 (41-57)
Group 3	71±5	26±2	9.6 (3-19)	11.7 (7-25)	50 (41-59)
Group 4	70±4	24±3	8.1 (3-14)	10.0 (6-16)	49 (34-61)
Group 5	71±4	28±4	10.2 (5-16)	13.0 (11-18)	45 (35-64)
Group 6	68±6	33±4	23.4 (18-30)*	23.9 (15.34)*	52 (41-73)
Group 7	64±7†	42±7*	26.7 (20-34)*	26.4 (17-34)*	57 (44-63)§

Values are median (range in parentheses) or mean±SE for Lm and CV. N= number of animals; Lm, mean linear intercept; CV, coefficient of variation; IS, bronchiolar injury score; A-A, percentage of ruptured bronchiolar-alveolar attachments; D_{A-A}, inter-attachment distance.. No significant differences occurred among groups 1 to 5, or between group 6 and 7. Significantly different from pooled groups 1 to 5: *P<0.001; §P=0.007; †P=0.025.

Table 3. Indices of parenchymal and vascular injury in normal open-chest rats mechanically ventilated with different tidal volumes

	Parameters	Injury score/ Number of rats			
		0	1	2	3
Group 1 & 2	focal alveolar collapse	18			
	perivascular edema	18			
	peribronchial edema	18			
	hemorrhage	18			
	alveolar granulocytes	18			
Group 3 & 4	focal alveolar collapse	17	1		
	perivascular edema	18			
	peribronchial edema	18			
	hemorrhage	18			
	alveolar granulocytes	18			
Group 5	focal alveolar collapse	7	2		
	perivascular edema	2	5	2	
	peribronchial edema	9			
	hemorrhage	9			
	alveolar granulocytes	9			
Group 6	focal alveolar collapse		3	6	
	perivascular edema			3	6
	peribronchial edema	6	2	1	
	hemorrhage	4	3	2	
	alveolar granulocytes		5	3	1
Group 7	focal alveolar collapse		2	6	1
	perivascular edema			5	4
	peribronchial edema	3	4	2	
	hemorrhage	2	2	3	2
	alveolar granulocytes		2	4	3

Injury score: 0=absent, 1=mild, 2=moderate, 3=marked.

Legends

Fig. 1. Time line representation of the main procedures used in rats mechanically ventilated on positive end-expiratory pressure (PEEP; 2.3±0.01 cmH_2O). Before (control condition) and after (test condition) 2 or 4 hrs of mechanical ventilation (MV) with the specified tidal volume (V_T; test period), the animals were mechanically ventilated with the baseline breathing pattern (BP). Broken and solid lines indicate PEEP and peak pressure (Ppeak) reached during MV. Hatched bars indicate when lung mechanics, arterial blood gases, and pH were assessed. Crossed bars correspond to the periods in which BALF and serum were collected, wet lung weight assessed and histologic procedures started.

Fig. 2. Group mean values of arterial pH (pHa), oxygen (Pa_{O_2}) and carbon dioxide partial pressure (Pa_{CO_2}), and systemic blood pressure (Pa) in open-chest rats during baseline mechanical ventilation before (control) and after (test) mechanical ventilation with different patterns (see Table 1). Bars: SE. * significantly different from control; § significantly different from preceding group.

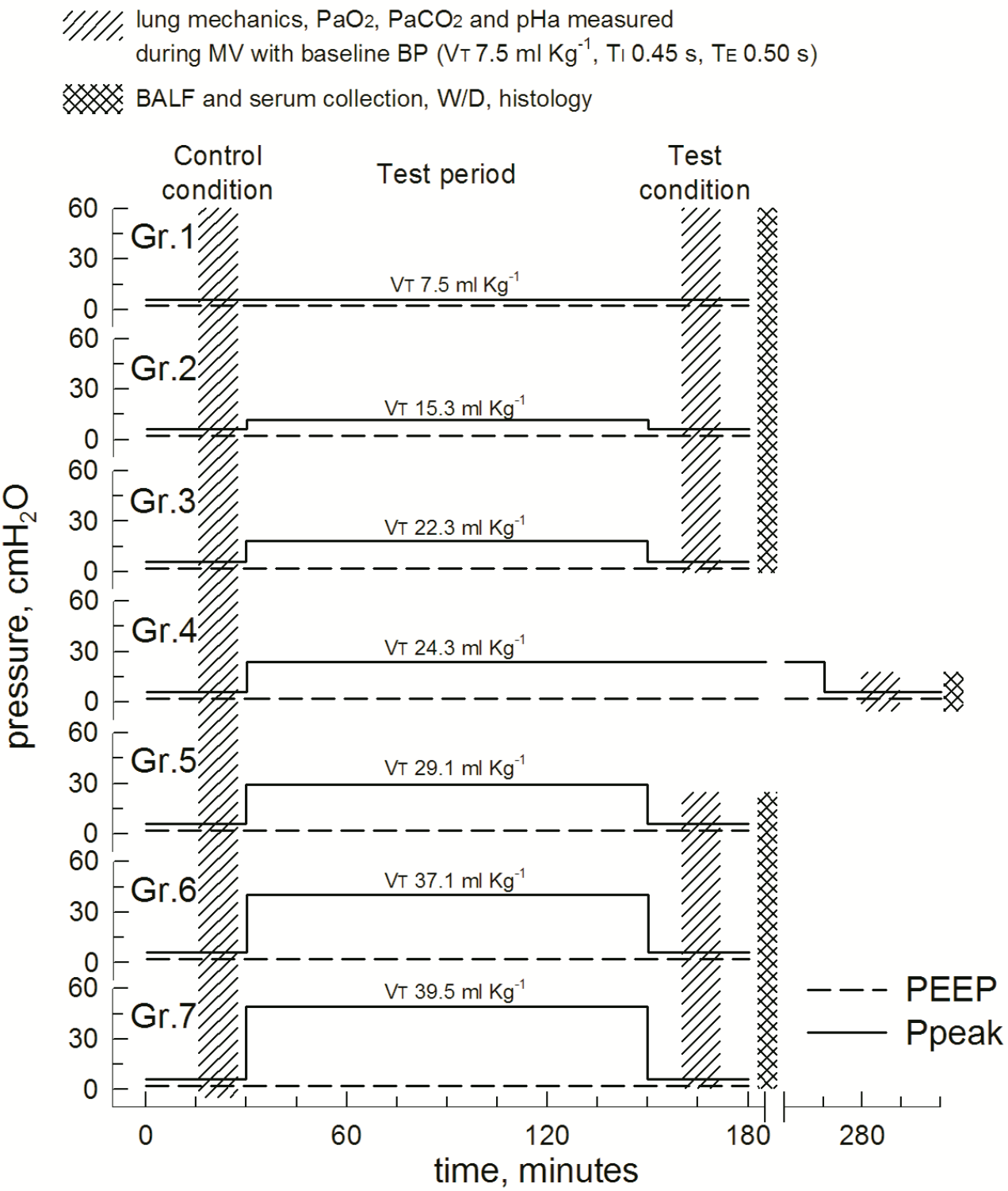
Fig. 3. *Upper diagrams*: group mean values of lung wet-to-dry weight (W/D) and bronchoalveolar lavage fluid to serum albumin concentration ratio (A_{BALF}/A_{SERUM}) in open-chest rats after mechanical ventilation with different patterns (see Table 1). Bars: SE. *significantly different from control; §significantly different from preceding group. *Lower diagrams*: W/D versus peak inflation pressure (Ppeak) during mechanical ventilation with different patterns (see Table 1) and W/D versus A_{BALF}/A_{SERUM} ratio at the end of the experiment in 63 open-chest subjected mechanical ventilation with different patterns (see Table 1). The dotted lines delimit the range (mean±3SD) of W/D and A_{BALF}/A_{SERUM} normal values (see text). Pcrit and W/Dcrit are values above which W/D and A_{BALF}/A_{SERUM} became higher than normal.

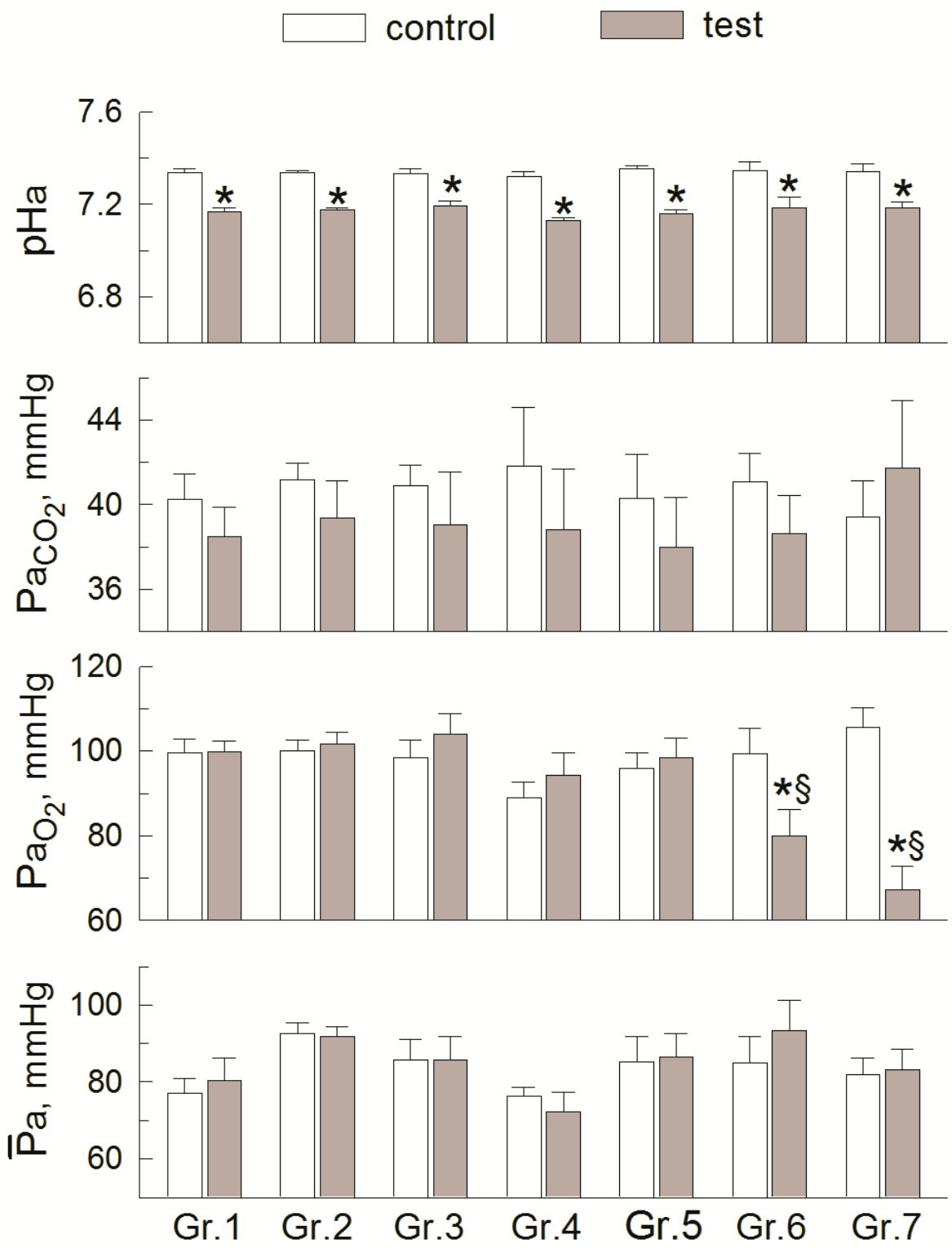
Fig. 4. Cytokine levels (median, 75th and 95th percentiles) of serum and bronchoalveolar lavage fluid (BALF) obtained in open-chest rats mechanically ventilated with different patterns (see Table 1). *significantly different from control; §significantly different from preceding group.

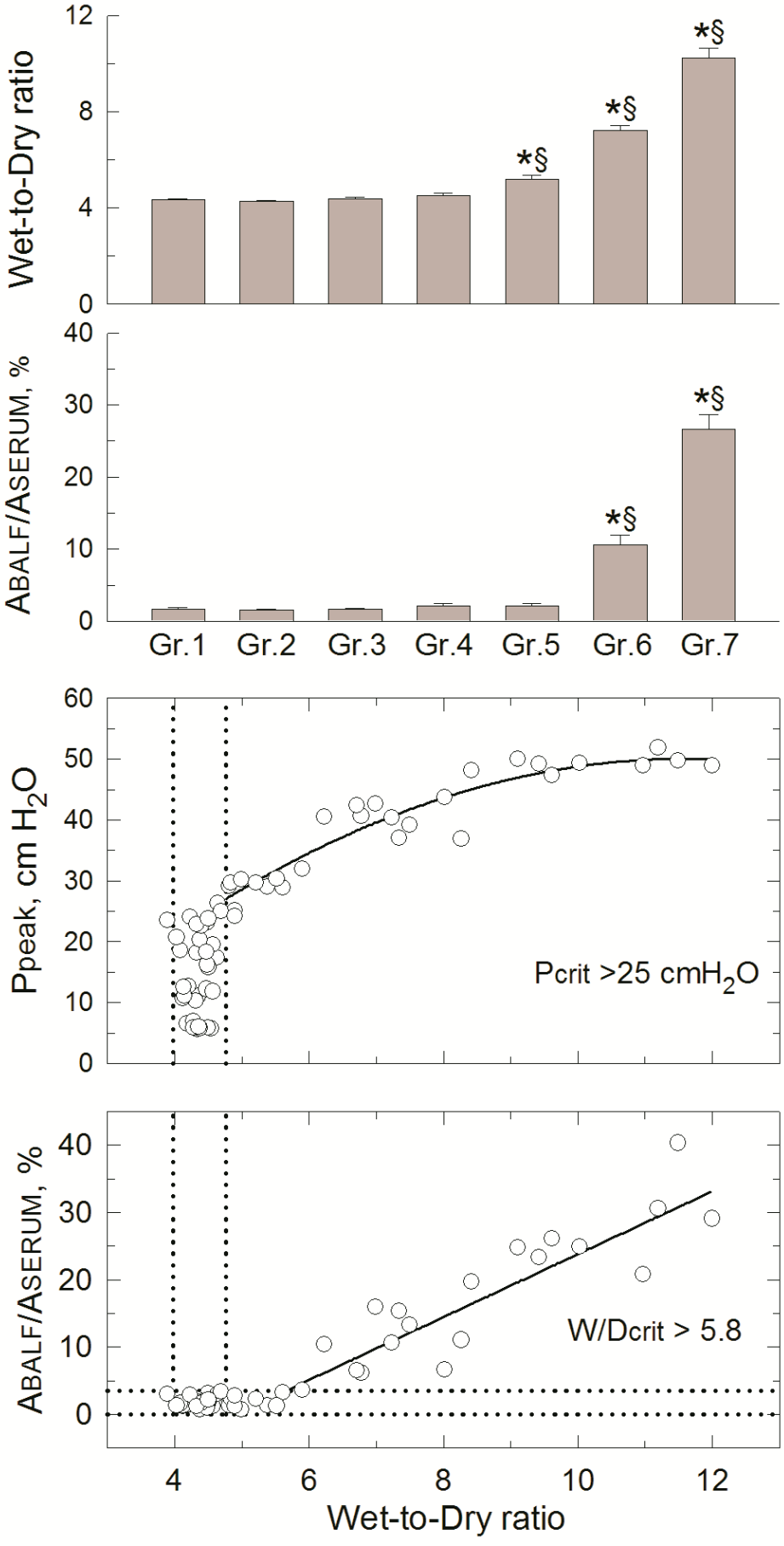
Fig. 5. Serum levels (median, 75th and 95th percentiles) of von Willebrand Factor (vWF) and E-selectin obtained in open-chest rats mechanically ventilated with different patterns (see Table 1). Individual values were expressed as percentage of the normal value (see text). *significantly different from normal; §significantly different from preceding group.

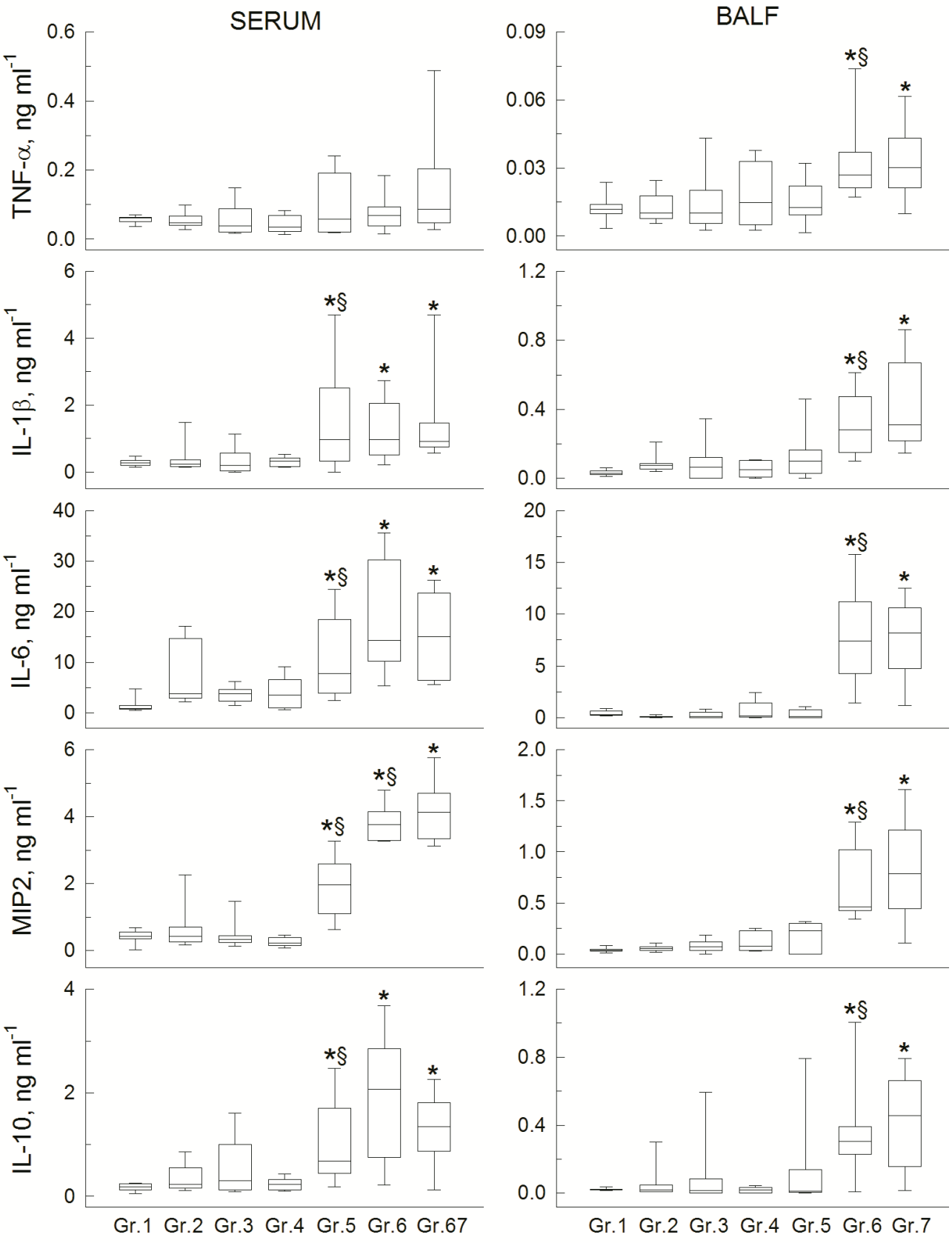
Fig. 6. Group mean values of the difference between the end-expiratory and the resting lung volume ($\Delta EELV$), lung quasi-static elastance (Est), interrupter resistance (Rint), viscoelastic resistance (Rvisc) and time constant ($\tau visc$) in open-chest rats during baseline mechanical ventilation before (control) after (test) mechanical ventilation with different patterns (see Table 1). Bars: SE. *significantly different from control; § significantly different from preceding group.

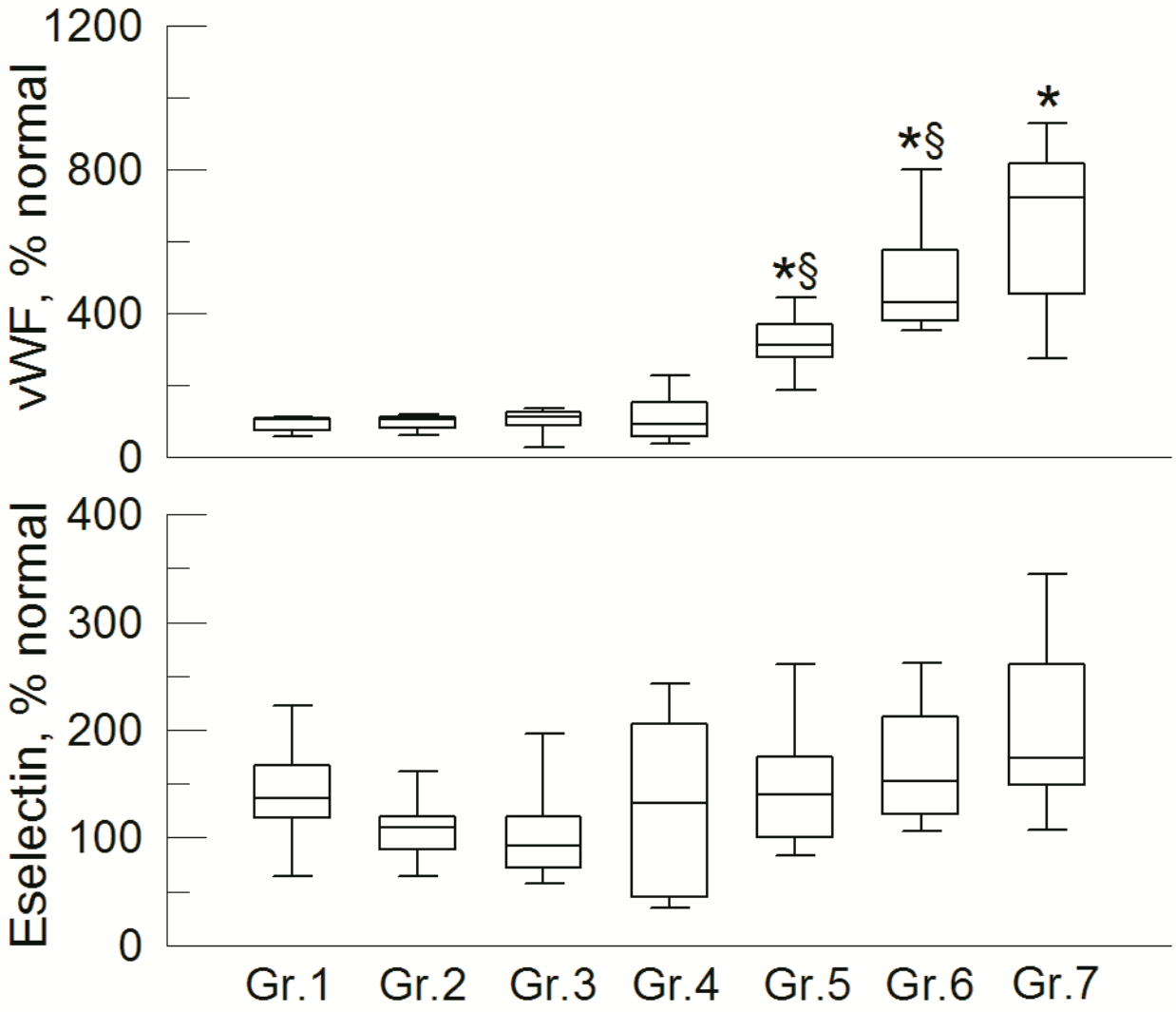
Fig. 7. Linear relationships between the changes of wet-to-dry lung weight (W/D) and those of quasi-static elastance (Est) or interrupter resistance (Rint), and between the changes in the percentage of ruptured broncho-alveolar attachments (A-A) and those of Rint in rats of group 6 and 7. Reference value for computing individual ΔEst and ΔRint or ΔA-A were the corresponding group 1-5 means or median, respectively. For individual ΔW/D the reference value was the corresponding group 1-4 mean (4.4±0.2) plus 3 times SD. Numbers indicate the slope (±SE) of the relationship.

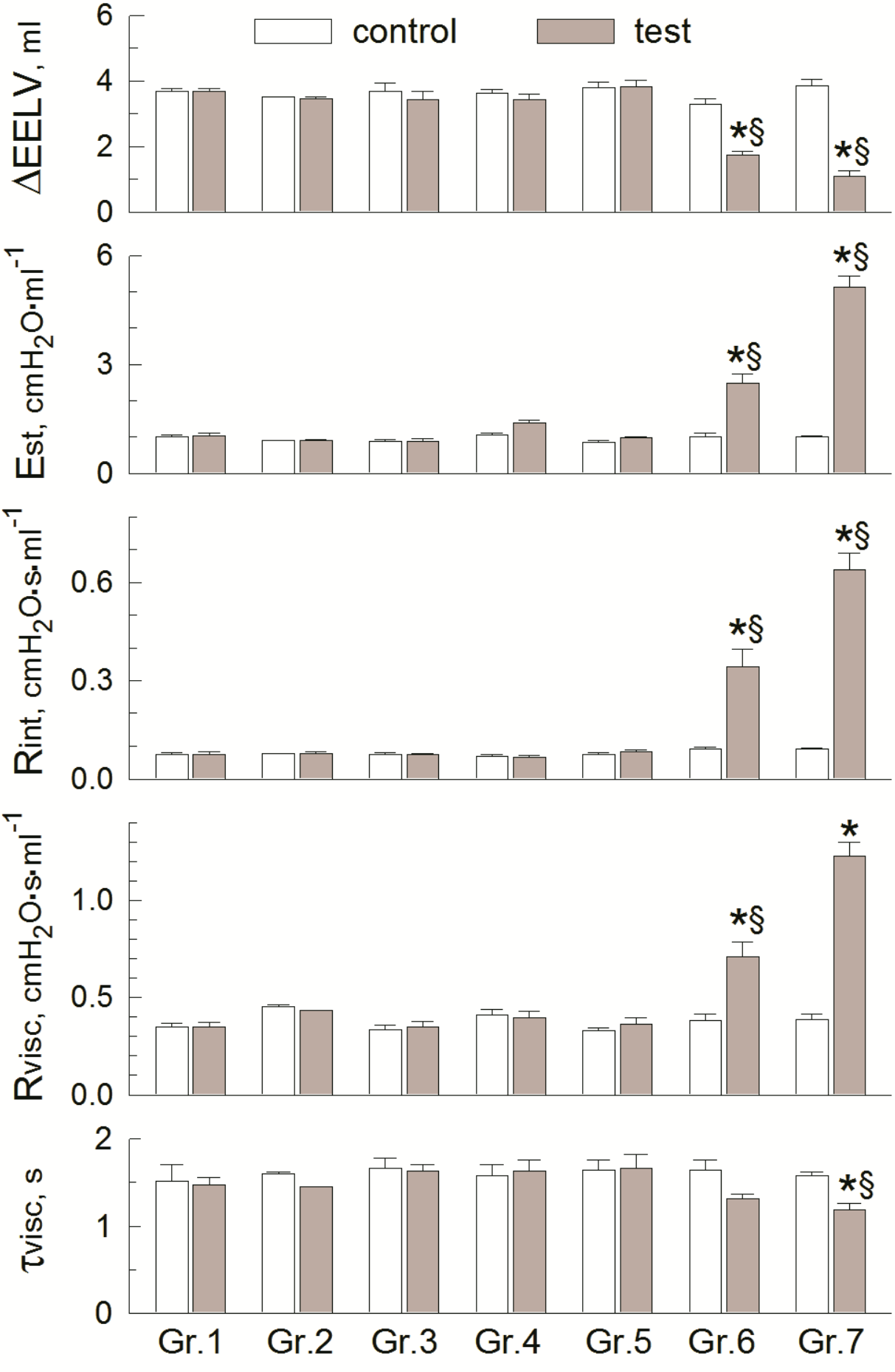

lung mechanics, PaO2, PaCO2 and pHa measured
during MV with baseline BP (VT 7.5 ml Kg-1, TI 0.45 s, TE 0.50 s)
BALF and serum collection, W/D, histology
Control condition
Test period
Test condition
Gr.1
VT 7.5 ml Kg-1
Gr.2
VT 15.3 ml Kg-1
Gr.3
VT 22.3 ml Kg-1
Gr.4
VT 24.3 ml Kg-1
Gr.5
VT 29.1 ml Kg-1
Gr.6
VT 37.1 ml Kg-1
Gr.7
VT 39.5 ml Kg-1
60
30
0
pressure, cmH2O
PEEP
Ppeak
0
60
120
180
280
time, minutes

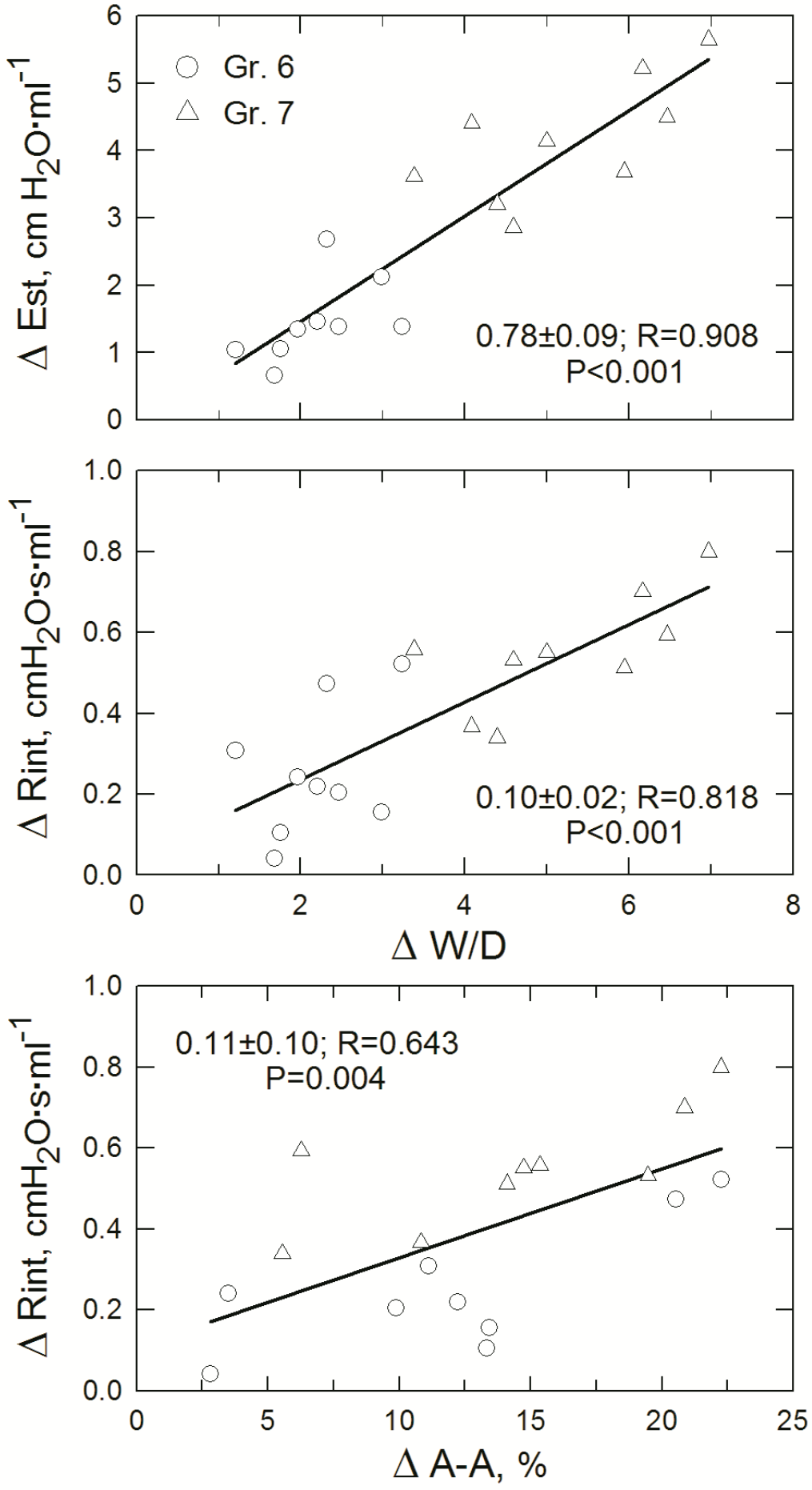

control
test
pHa
7.6
7.2
6.8
Pa_{CO_2}, mmHg
44
40
36
Pa_{O_2}, mmHg
120
100
80
60
$\bar{P}a$, mmHg
100
80
60
*
*§
Gr.1
Gr.2
Gr.3
Gr.4
Gr.5
Gr.6
Gr.7


Wet-to-Dry ratio
12
8
4
0
*§
*§
*§
ABALF/ASERUM, %
40
30
20
10
0
*§
*§
Gr.1
Gr.2
Gr.3
Gr.4
Gr.5
Gr.6
Gr.7
Ppeak, cm H2O
60
50
40
30
20
10
0
Pcrit >25 cmH2O
ABALF/ASERUM, %
40
30
20
10
0
W/Dcrit > 5.8
4
6
8
10
12
Wet-to-Dry ratio


SERUM
BALF
TNF-α, ng ml-1
IL-1β, ng ml-1
IL-6, ng ml-1
MIP2, ng ml-1
IL-10, ng ml-1
Gr.1 Gr.2 Gr.3 Gr.4 Gr.5 Gr.6 Gr.67
Gr.1 Gr.2 Gr.3 Gr.4 Gr.5 Gr.6 Gr.7


vWF, % normal
1200
800
400
0
*§
*§
*
Eselectin, % normal
400
300
200
100
0
Gr.1
Gr.2
Gr.3
Gr.4
Gr.5
Gr.6
Gr.7


control
test
ΔEELV, ml
Est, cmH2O·ml-1
Rint, cmH2O·s·ml-1
Rvisc, cmH2O·s·ml-1
τvisc, s
*§
*
Gr.1
Gr.2
Gr.3
Gr.4
Gr.5
Gr.6
Gr.7


Gr. 6
Gr. 7
0.78±0.09; R=0.908
P<0.001
Δ Est, cm H2O·ml-1
0.10±0.02; R=0.818
P<0.001
Δ Rint, cmH2O·s·ml-1
Δ W/D
0.11±0.10; R=0.643
P=0.004
Δ Rint, cmH2O·s·ml-1
Δ A-A, %