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A simple method for the differential characterization of alveoli and alveolar ducts in injured lungs

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Summary. Rationale and hypothesis: Previous studies evaluating the histoarchitecture of distal airspaces have been shown to be limited by the difficulty in adequately differentiating alveoli and alveolar ducts. This limitation has been specially noticed in studies addressing lung recruitment and in situations of diffuse alveolar damage (DAD), where generic nominations for distal airspaces had to be created, such as "peripheral airspaces" (PAS) and "large-volume gas-exchanging airspaces" (LVGEA). Elastic stains have been largely used to describe normal lung structures. Weigert's resorcin-fuchsin staining (WRF) demarcates the thickened free portions of the ductal septum facilitating its recognition. We hypothesized that this staining could help in differentiating alveoli from alveolar ducts in distorted lung parenchyma. Material and Methods: Samples of control lungs and of DAD lungs induced by mechanical ventilation (VILI) were stained with hematoxylin-eosin (H&E) and with WRF. Using morphometry we assessed the volume proportion of alveoli, alveolar ducts and LVGEA in control and VILI lungs. Results: WRF stained VILI lungs showed a significant decrease in the volume proportion of LVGEA and alveoli and a significant increase in the volume proportion of alveolar ducts when compared to H&E stained samples. Conclusion: We conclude that WRF staining is useful to distinguish alveolar ducts from alveoli in a DAD model, and suggest that it should be routinely used when morphometric studies of lung parenchyma are performed.

Key words: Extracellular matrix, Elastic tissue, Diffuse alveolar damage, Respiratory mechanics, Morphometry

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Introduction

The distal end of the airway tree is called the respiratory zone, where the diffusion of gases takes place. It includes the alveoli, which are limited by septa and disposed around air-supplying channels, called alveolar ducts, which are intimately related to the alveoli and are sometimes difficult to distinguish from them (Weibel and Taylor, 1998). Alveolar septa consist of a net of capillaries surrounded by a thin layer of connective tissue fibers. At the free distal end of the ductal septa, the frame of fibers is reinforced and forms the "alveolar mouths" delimitating the alveolar ducts (Soubin et al., 1988; Mercer and Crapo, 1990).

Many morphometric studies have been conducted to address the behavior of distal airspaces during the respiratory cycle in normal and damaged lungs (Gil and Weibel, 1972; Gil et al., 1979; Silva et al., 1998; Escolar et al., 2002). The evaluation of the histoarchitecture of distal airspaces at different lung volumes has been shown to be limited by the difficulty in adequately differentiating alveoli and alveolar ducts (Gil et al., 1979; Escolar et al., 2002). In fact, due to this difficulty, Escolar et al. (2002) have used the term "peripheral airspaces" (PAS) as a generic nomination for ducts and alveoli in a morphometric study describing alveolar recruitment in normal lungs. Gil et al. (1979) stressed that at a low inflation degree, "it was often impossible to draw an unambiguous demarcation line between alveolar ducts and alveoli" in normal rabbit lungs fixed at different volumes. This limitation has been especially noticed in a situation of diffuse alveolar damage as edema, inflammation and tissue distortion prevail (Silva et al., 1998). In damaged lungs, airspaces larger than normal alveoli are frequently observed, and may actually represent distended alveolar ducts or hyperinflated alveoli. Due to a lack of precision and to the subjectivity of the observer, it was deemed suitable to classify such structures as large-volume gas-exchanging airspaces (LVGEA) (Silva et al., 1998).

Connective tissue stains have been largely used to describe normal lung structures. Weibel and Taylor (1998) described how the alveolar ducts carry fibers of the axial fiber system in their walls, which extend to the end of the duct system. These fibers encircle the alveolar mouths forming rings that serve as a scaffold for a network of finer fibers that spread within the alveolar septa.

In previous studies with lung tissue obtained from patients who died of Acute Respiratory Distress Syndrome, it was observed that the cuff of elastic fibers involving the alveolar ducts was easy to identify if properly stained, even in a situation of alveolar damage (Negri et al., 2000). Based on this observation, we hypothesized that the identification of the elastic fiber distribution within the lung parenchyma could facilitate the proper classification of the distal airspaces even in pathological conditions. We believe that if this hypothesis is confirmed, a simple and non-expensive staining method could then be used to improve the observations obtained at morphometric studies of distal airspaces, since these studies are usually conducted with Hematoxilin-Eosin (H&E) stained slides (Gil and Weibel, 1972; Gil et al., 1979; Silva et al., 1998; Escolar et al., 2002). Therefore, the aim of the present study was to demonstrate that the use of a widely known staining method for elastic fibers helps to objectively distinguish alveolar ducts from alveoli in a model of diffuse alveolar damage.

Material and methods

Inducing VILI

Ten rats of similar weight (277 to 340 g) were divided in two groups: control and Ventilator Induced Lung Injury (VILI) (Mori et al., 2002). All animals were anesthetized by intra-peritoneal administration of ketamine chloridrate 8 mg/kg and xylazine 0.4 mg/kg and paralyzed using pancuronium bromide (0.2 mg/kg). Animals were then submitted to a tracheostomy and connected to a mechanical ventilator (Inter 3, Intermed Equip., São Paulo, Brazil).

The control group was submitted to mechanical ventilation using a tidal volume of 10 ml/kg of body weight, a positive end expiratory pressure (PEEP) of 5 cm H_2O and 100% FIO_2, at peak inspiratory pressures that varied from 10 to 15 cm H_2O, for twenty minutes. Ventilation Induced Lung Injury was induced by ventilating the rats with a tidal volume of 45 ml/kg of body weight at 5 cm H_2O of PEEP and FiO_2 of 100%, at peak inspiratory pressures that varied from 50 to 60 cm H_2O (high-peak pressure mechanical ventilation). The use of high-peak pressure mechanical ventilation has been shown to cause alveolar edema and parenchymal changes consistent with Diffuse Alveolar Damage (DAD), and was therefore applied to induce lung injury (Dreyfuss et al., 1988). Respiratory system elastance and resistance were measured by the end inflation occlusion method (Martins et al., 1989) every five minutes. The time of ventilation in the VILI group varied among the animals, and was maintained until an increment of 30% of the respiratory system elastance, which should monitor the installation of lung parenchyma injury, was observed.

Perfusion and fixation of the lungs

Rats were maintained connected to the ventilator, then they were submitted to a median laparotomy and the vena cava was cannulated. A perfusion solution consisting of physiological saline saturated with 2.2 mM $CaCl_2$, 0.5% procaine and heparin (25,000 IU/L) was infused through the vena cava. Simultaneously, the aorta was sectioned and the infusion was maintained until the drainage fluid was clear. Then, the lungs were fixed by perfusion via vena cava using 15 ml of a fixative solution, consisting of 3.6% formaldehyde and 0.25% glutaraldehyde for 10 minutes (Gil and Weibel, 1972). The perfusion pressure was maintained at 20 cmH_2O. Following this procedure, the trachea was clamped and the thorax was opened. The lungs were removed en bloc and immersed in the fixative solution. After 24 hr, 3 sagittal sections were performed in the left lung, one adjacent to the hilus, and the other two 1.0 cm apart towards the pleural surface. The middle slice was used for morphometrical measurements, as it represents the largest surface of the lung. Lung sections were embedded in paraffin and routinely processed. Two 5 mm-thick histological section slides were made from each sample block. One of them was stained with hematoxylin and eosin (H&E) and the other with Weigert's resorcin-fuchsin method (WRF) for the identification of elastic fibers (Weigert, 1898; Montes, 1996). The WRF method selectively stains the three types of elastic fibers (oxytalan fiber, elaunin fiber, and fully developed elastic fiber), but does not allow their differentiation (Fullmer et al., 1974; Montes, 1996).

Classification of distal airspaces

Based on classical morphological studies, alveoli were defined as hexagonal structures limited by continuous walls (interalveolar septa). Alveolar ducts were defined as airway spaces limited by free tips of interalveolar septa (alveolar "mouth") (Whimster, 1975). Previous studies using thick serial sections have shown that smooth muscle and accompanying bundles of collagen and elastic fibers spiral down the alveolar ducts like a spring (Pierce and Eber, 1965), which can facilitate their recognition. When the anatomical classification of distal airspaces as alveoli or alveolar ducts was not possible due to overdistension or tissue distortion, these peripheral airspaces were classified as large-volume gas-exchanging airspaces (LVGEA) (Silva

et al., 1998).

Morphometry

Distal airspaces were quantified by the conventional morphometric method of point counting, using a 100-point grid of known area coupled to the optical microscope (Gil and Weibel, 1972). The volume proportion of alveoli, alveolar ducts and LVGEA was assessed by determining respectively the number of points hitting alveoli, alveolar ducts and LVGEA lumen in 6 non-overlapping randomly selected fields in each lung sample, using a magnification of 100x.

According to Weibel and Cruz-Orive (1997), the relative section area occupied by a given structure corresponds to its volume density in the total tissue volume in a statistical sense, i.e., "the area density of a given structure is an unbiased estimator of its volume density. If we place a set of test points onto the section, each point hits the structure in proportion to its areal or volume density". The number of points in the grid is directly proportional to its area. Therefore, 100 points correspond to the total area of the grid. By determining the number of points hitting each parenchymal structure (alveolar ducts, alveoli and LVGEA), it is possible to calculate the volume proportion of these structures in the overall lung parenchyma, expressed as a percentage area (Weibel, 1979). The volume proportion of alveolar ducts (Vad), alveoli (Valv) and LVGEA (Vlvgea) in the lung parenchyma was then calculated as follows:

(1) Vad = number of points hitting alveolar duct lumen
(2) Valv = number of points hitting alveolar lumen
(3) (Vlvgea) = number of points hitting LVGEA lumen

One value of Vad, Valv and Vlvgea was calculated for each field. Volume proportion was then expressed as a percentage.

Morphometric measurements were performed in both H&E and WRF stained slides. Therefore, all fields were analyzed in the same slide for each staining, avoiding double counting.

The selection of parenchyma regions to be analyzed, as well as the point counting, were performed by a blinded investigator. Interobserver comparisons were performed by 2 investigators. The coefficient of variation for the interobserver error for point counting was < 5%.

Statistical analysis

Statistical analysis was performed using the non-parametric Mann-Whitney test for comparison between H&E and WRF groups in control and VILI lungs. Statistical significance was set at $p<0.05$.

Results

Control lungs showed preserved parenchyma architecture and did not present any sign of lung disease, such as edema, collapse or inflammation. VILI lungs presented areas of collapse as well as overdistention, alveolar edema, patchy hemorrhage, and mild interstitial inflammation.

Figure 1 shows the histological picture of control and VILI lungs. Non-classifiable large distal airspaces (LVGEA) are seen in H&E-stained slides in VILI lungs. A distinction between alveolar ducts and alveoli was facilitated by WRF in both normal and injured lungs, as the cuff of elastic fibers is reinforced at the alveolar ducts "mouths".

Table 1 shows morphometric data of all classified structures in the 2 groups. Data are expressed as median and range. In normal lungs, there was no significant difference in the volume proportion of either parenchymal structure when H&E was compared with WRF stained slides. WRF stained VILI lungs showed a significant decrease in the volume proportion of LVGEA ($p=0.036$) and alveoli ($p=0.013$) and a significant increase in the volume proportion of alveolar ducts ($p<0.001$) when compared with H&E stained samples.

Figure 2 shows LVGEA data in control and VILI lungs. In control lungs alveoli can be easily differentiated from alveolar ducts, with few structures classified as LVGEA and no significant difference in the LVGEA counts between H&E and WRF stained slides. However, in injured lungs, many of the structures that would be classified as LVGEA at H&E stained lungs are classified as either alveoli or alveolar ducts once WRF is

Table 1. Morphometric data of distal lung parenchyma. Data represents percentage of points hitting each studied structure in control and VILI lungs stained by H&E and WRF method. Data is expressed as median and range.

	CONTROL H&E	CONTROL WRF	VILI H&E	VILI WRF
Alveolar ducts	71±37	77±35.8	55±14	66±4.1
Alveoli	7.8±4.5	8.6±9.0	9.6±13	7.6±9.5
LVGEA	0.1±5.3	0.3±3.5	3.8±22	0.8±9.8

H&E: hematoxylin and eosin staining. WRF: Weigert´s resorcin-fuchsin staining. VILI: Ventilator Induced Lung Injury.LVGEA: Large-Volume Gas-Exchanging Airspaces

applied, with a significant decrease in LVGEA counts.

Discussion

In the present study, we demonstrated that the use of a simple and widely known staining method can greatly improve the accuracy of the identification of anatomical structures in the respiratory portion of injured lungs. Elastic staining has been largely used to describe tissue structures. In the skin, WRF staining has been used to stain the thick and intermediate diameter fibers of the dermis, as well as very thin superficial fibers in the basement membrane (Junqueira and Carneiro, 2003). In an interesting study, Montes et al. (1985) showed that the WRF method could be useful for the delineation of the elastic fibers in assessing the histoarchitecture of organs that have undergone considerable distortion, such as in mummified tissues. The alveolar ducts carry elastic fibers in their walls, which encircle the alveolar mouths (Weibel and Taylor, 1998). In the present study we have shown that the staining of these fibers can help differentiate distal lung parenchymal structures, even when there is lung tissue distortion.

When morphometry was first applied to the study of distal lung parenchyma, many studies were carried out in order to explain how the lung opens and how it recruits its units. Forrest (1970) suggested that there is no change in alveolar diameter in lung recruitment and that the

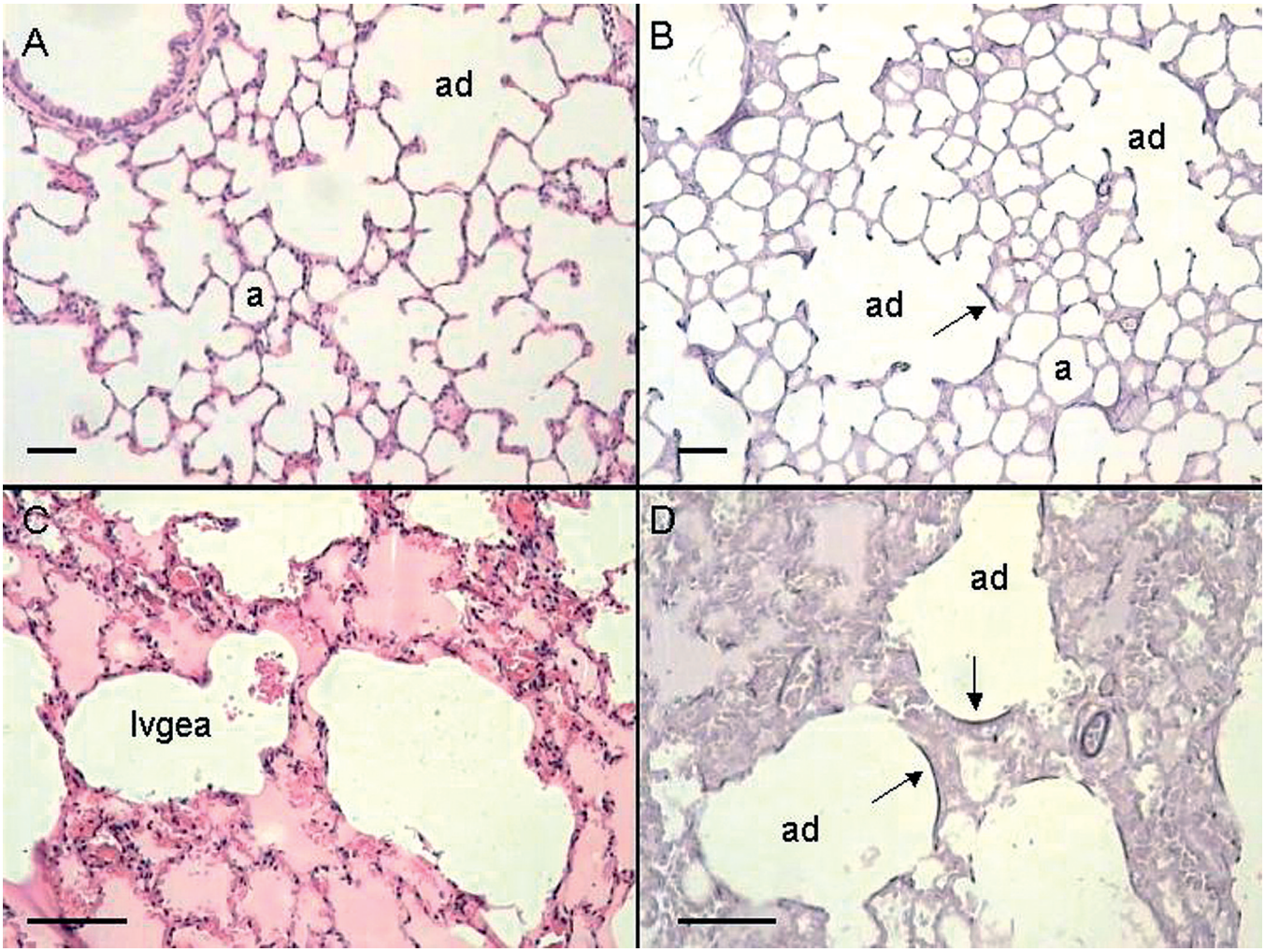

Fig. 1. Photomicrographs of control and injured lungs. **A**. Control lung stained by H&E. Note the preserved histoarchitecture of distal lung parenchyma, where it is possible to identify alveoli (a) and alveolar ducts (ad). **B.** Control lung stained with Weigerts's resorcin-fucsin (WRF). The arrow indicates the elastic fibers around the entrance of alveolar ducts (ad), facilitating its differentiation from alveoli (a). **C.** VILI lung stained with H&E showing alveolar edema and distortion of the parenchymal structures. Observe the large distended air spaces (lvgea) that cannot be easily classified. **D**. WRF stained VILI lung reveals that some distended air spaces can be identified as alveolar ducts (ad) by the presence of a cuff of elastic fibers (arrows) at the alveolar "mouths". The identification of alveolar ducts results in a decrease in the number of parenchymal structures that cannot be accurately

alveolar duct is the main structure involved in lung volume changes. Contrary to this hypothesis, Gil and Weibel (1972) have suggested that not only alveolar ducts, but also the alveoli are deformed and recruited during changes in lung volume. However, it is important to notice that, even when performing such studies in normal lungs, where it is easier to recognize and classify distal lung structures, the authors had some difficulty in accurately classifying structures in the distal lung parenchyma (Gil et al., 1979). More recently, Escolar et al. (2002) have shown that increase/decrease in pulmonary volume is mainly due to recruitment/de-recruitment of the peripheral airspaces. These authors also reported the difficulty in differentiating alveoli from alveolar ducts and used the term "peripheral airspaces" (PAS) to denominate either alveoli or alveolar ducts in histological sections of the lung periphery. The Acute Respiratory Distress Syndrome (ARDS) represents a global response of the lung parenchyma to a variety of insults that culminates in a diffuse alveolar/capillary injury, clinically characterized by the rapid onset of respiratory insufficiency. Different ventilatory strategies have been designed to minimize lung injuries and reduce pulmonary complications in these patients (Amato et al., 1998). Understanding distal lung parenchyma plasticity should certainly provide the tools to ameliorate the mechanical ventilation settings in situations of acute lung injury. Although computed tomography has significantly contributed to the study of lung behaviour in ARDS (Crotti et al., 2001), histological studies are essential to understanding lung recruitment at different pressures imposed to damaged lungs.

The histological classification of parenchymal structures in injured lungs is much more difficult than in normal lungs, as inflammation, collapse and overdistension alter the lung tissue architecture. In an attempt to evaluate the histoarchitecture of distal airspaces in acute lung injury, Silva et al. (1998) had to create a new nomenclature in order to denominate large distended parenchymal structures, since it was not possible to accurately determine the differences between distended alveoli and alveolar ducts in histological sections. In the present study, we aimed to demonstrate that WRF, a widely known staining method for elastic tissue, could be used to facilitate the differentiation of alveoli and alveolar ducts in damaged lungs. To perform such analysis, we have chosen the high-pressure mechanical ventilation model of lung injury (VILI), as it leads to an increase in endothelial and epithelial permeability, with consequent diffuse tissue damage (Dreyfuss et al., 1998; Mori et al., 2002). Our results show that staining the elastic tissue in injured lungs is useful to obtain a more precise classification of distal airspaces, with a consequent decrease in the number of parenchymal structures that cannot be accurately classified. These results have practical implications, since they suggest that it is possible to improve the observations drawn from morphometric studies of distal airspaces, even in pathologic situations. We believe that the application of this method could be of great value for the full comprehension of the relationship between ducts and alveoli during the inflation and deflation of lung parenchyma in health and disease, adding to the knowledge of the mechanisms related to lung recruitment during mechanical ventilation.

In conclusion, we have shown that Weigert's resorcin-fuchsin method is a useful technique to distinguish alveolar ducts from alveoli in a diffuse alveolar damage model, and suggest that it should be routinely used (in addition to hematoxylin-eosin staining) when morphometric studies of distal lung parenchyma are performed.

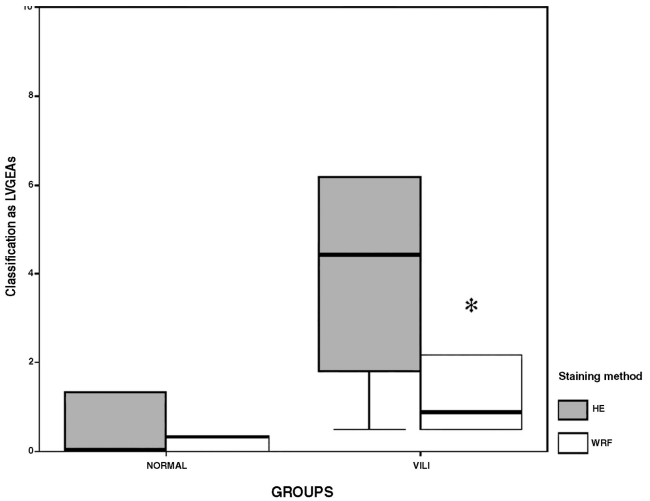

Fig. 2. The graph shows the volume proportion of LVGEA in control and VILI lungs stained by H&E and WRF methods. The box plots represent the median and interquartile range of the morphometric data. There was a significant decrease (*) in LVGEA counts in injured lungs when WRF staining method was applied, compared with H&E staining (p=0.036). This difference was not observed in normal lungs.

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