Variation of lung volume after fixation when measured by immersion or Cavalieri method

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Yan, Xiao, Juan Jose Polo Carbayo, Ewald R. Weibel, and Connie C. W. Hsia. Variation of lung volume after fixation when measured by immersion or Cavalieri method. Am J Physiol Lung Cell Mol Physiol 284: L242–L245, 2003. First published September 13, 2002; 10.1152/ajplung.00184.2002.—Organ volume is a critical parameter in morphometric analysis. The special problems of the lung as a nonsolid organ are overcome by tracheal instillation of fixatives at a constant airway pressure (Paw). Lung volume can change significantly after fixation as Paw change. To determine the variation of lung volume after fixation, we measured the volume of intact fixed lungs by serial immersion in saline (Vimm) at selected time points, compared with measurements obtained by point counting [Cavalieri Principle (Vcav)] after tissue sectioning to release Paw. Vimm was systematically higher than Vcav by 25% in dog lungs and 13% in guinea pig lungs (P = 0.0003 between species). This size-dependent variability reflects residual elastic recoil, refolding and/or crumpling of alveolar septa after fixation. Vimm remained 14% higher than Vcav in dog lungs after pressure release. Vcav/Vimm was systematically lower in the upper than the lower strata of the same lung. We conclude that Vcav measured on lung slices after relaxation of Paw more precisely represents the state of the tissue to be used for subsequent morphometric analysis, particularly for large lungs.

tracheal instillation; saline immersion; Scherle method; point counting; dog; guinea pig; morphometry

Because the volume of an inflated lung comprises ~90% air and 10% tissue, considerable problems arise when lung tissue must be prepared for morphometric analysis. Lung tissue should ideally be fixed in such a way as to 1) accurately reflect the physiological state of inflation and perfusion and 2) resist distortion by further processing for microscopy. The tissue must also be made stiff enough to allow the organ volume to be measured as a baseline parameter for the calculation of morphometric data representing the whole lung. To achieve this, a common method of lung fixation is the intratracheal instillation of glutaraldehyde fixatives at a constant hydrostatic pressure, usually 20 to 25 cmH2O above the highest point of the sternum. After instillation, the trachea is clamped to maintain the airway pressure, and the lung is immersed in fixatives for at least several days before sectioning and histological processing. Because elastin is not fully stiffened by glutaraldehyde (11), the fixed lung continues to exhibit residual elastic recoil and may shrink in volume when eventually sectioned. We consistently observed such shrinkage to occur in fixed dog lungs (5, 16). Because lung volume is a basic measurement and the only absolute value used in calculating cellular and tissue compartment volumes and surface areas from microscopic estimations of volume and surface densities (17, 18), it is imperative that the magnitude and sources of variability in this measurement be understood.

In this study, we examined the magnitude of pressure-related change in lung volume after fixation of dog and guinea pig lungs by tracheal instillation of a glutaraldehyde solution under controlled airway pressure. At different time points after fixation, we measured the volume of the fixed lobes by 1) saline immersion (19) and 2) point counting after serial sectioning to release airway pressure (Cavalieri method) (18). We expected similar results by the two techniques if lung elastic recoil is eliminated by fixation. On the other hand, a systematically larger lung volume measured by immersion would indicate residual elastic recoil of the fixed alveolar septa. We compared dog and guinea pig lungs to determine whether lung size affects the pressure-related volume change after fixation.

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METHODS

Lung fixation. We retrospectively analyzed lung volume data from experimental animals used as part of other published (3–5, 16) and ongoing studies. All protocols had been approved by the Institutional Animal Care Research and Advisory Committee. The animals included 1) normal foxhounds (3 mo to 2 yr of age, 5.7–30 kg body wt), 2) foxhounds of similar ages at various time points after undergoing pneumonectomy, and 3) normal male guinea pigs 2–8 mo of age (0.4–1.0 kg body wt). At the time of euthanasia, the dog was deeply anesthetized with intravenous Nembutal (25 mg/kg) and placed in a supine position. Through a tracheostomy, auffed endotracheal tube was inserted. After the cuff was inflated, the endotracheal tube was securely tied to the trachea using umbilical tape. The dog was mechanically ventilated at a tidal volume of 12 ml/kg. In some animals, the abdomen was opened via a midline incision. A cut was made through each hemidiaphragm just below the xiphoid process to expose both hemithoraces. In other animals, an incision was made through a lateral intercostal space on each side to open the pleural space. In either case, the ventilator was stopped allowing the lung(s) to collapse. Then the lungs were reinfated within the chest via tracheal instillation of 2.5% buffered glutaraldehyde at a constant hydrostatic pressure (25 cmH2O above the highest point of the sternum). After the flow of fixative stopped, the tracheal tube was clamped to maintain the pressure within the airways. The chest was opened and the lungs were dissected en bloc, immersed in 2.5% buffered glutaraldehyde in a plastic bag, which was floated on a water bath, and stored at 4°C until further processing.

The guinea pigs were anesthetized with an intraperitoneal injection of ketamine and xylazine. A tracheostomy was performed. A piece of silastic tubing attached to a Luer lock connector was inserted and tied securely to the trachea by silk sutures. The animal was mechanically ventilated at a tidal volume of 12 ml/kg. Through a lateral thoracotomy, the left hilum was tied with silk suture and the left lung was removed for other studies. The ventilator was stopped to allow the right lung to collapse. Then the right lung was reinfated by intratracheal instillation of 2.5% buffered glutaraldehyde at 25 cmH2O hydrostatic pressure above the sternum. After the tracheal tube was clamped, the lungs were removed from the chest, floated on a bath of 2.5% buffered glutaraldehyde, and stored at 4°C until further processing.

Lung volume measurement. The fixed dog lungs were separated into left and right sides by clamping each main stem bronchus. Each lung was further separated into upper and lower strata. The upper stratum consisted of the upper and middle lobes, which were often partially connected. The lower stratum consisted of the lower lobe and cardiac lobe (on the right side). Volume of each stratum was measured by the immersion method of Scherle (19). A container of isotonic saline was placed on a weighing scale. After reading an initial weight (W0), the lung or stratum was totally submerged in saline without touching the sides and the final weight (Wf) was recorded. The difference in weight (Wf - W0 in g) was an estimate of volume (in ml); the specific gravity of isotonic saline (1.0048) was taken to approximate that of water (1.0). The entire right lung of guinea pigs was immersed. Measurements were made with the tracheal clamp intact to maintain airway pressure. In some lungs, measurements were repeated after opening the tracheal clamp to allow escape of fluid and equilibration of pressure.

After measuring the intact volume by immersion, each stratum of dog lung was sliced serially at 2-cm intervals using an electric knife, with the first cut placed at random, i.e., systematic sectioning with random start. Guinea pig lungs were serially sliced at 3-mm intervals using a customized sectioning device. The face of each section was photographed by a 35-mm Nikon camera using Kodak tungsten color film. A volume estimate of the sectioned lung was obtained from the photographs by point counting using the Cavalieri Principle (9). A grid was laid over the image and the points falling on lung (Pv) were counted. Images were analyzed at a magnification sufficient to yield at least 200 counted points per stratum. The volume (V) is calculated as

\[ V = \sum P_i \cdot \left( \frac{d}{m} \right)^2 \cdot t \]

where \( \sum P_i \) = sum of the points falling on the lung from all slices, \( t = \) section thickness, \( m = \) magnification, and \( d = \) distance between grid points (mm).

Temporal change in lung volume after fixation. In 15 intact dog lungs or strata stored for a prolonged time, we repeated immersion volume measurements at different time points up to 3 mo after fixation. After the final measurement, the lung was immediately sectioned and the volume after sectioning was determined by Cavalieri Principle as described above. In other dog lungs and in all guinea pig lungs, volume was measured by immersion once, followed immediately by sectioning. The interval between fixation and final lung sectioning and volume measurement ranged from 9 days to 3 mo for dog lungs and was 3 mo for guinea pig lungs. Results obtained by the two methods were expressed as the ratio of Cavalieri volume to the immersion volume estimated on the same day (Vcav/Vimm).

Data analysis. In dog lungs, measurements from upper and lower strata of the same animal were compared by paired t-test. Combined measurements from all strata were compared with those from guinea pig lungs by one-way analysis of variance.

RESULTS

Figure 1 shows the cumulative change in volume of intact fixed dog lungs measured by serial saline immersion at different time points. Beyond the first week after fixation, volume shrinkage was minimal as airway pressure was maintained; the actual pressure was
not known but it presumably was lower than the instillation pressure that prevailed at the first measurement point. The average changes in immersion volume of dog lungs followed for different times range from −3.3 to −7.1% of the initial volume estimated at 1 wk after fixation. The average ratios of Cavalieri-to-immersion volume estimation ($V_{cav}/V_{imm}$) in dog and guinea pig lungs at the time of sectioning are shown in Table 1. Volume measured without releasing airway pressure was 25% higher in both groups of dogs and 13% higher in guinea pigs compared with volume measured after tissue sectioning. The difference in volume reduction between dogs and guinea pigs is highly significant ($P < 0.0003$). In both pneumonectomized and sham dogs, the $V_{cav}/V_{imm}$ ratio was systematically and significantly lower in the upper stratum than the lower stratum (Table 1), indicating greater shrinkage of the upper stratum with release of pressure.

In three additional dogs, lung volume was measured by immersion after release of the tracheal clamp to equilibrate airway pressure to atmosphere. The ratios of Cavalieri-to-immersion volume ($V_{cav}/V_{imm}$) in these animals were 0.91, 0.83, and 0.83 (mean 0.86), i.e., volume estimated by immersion was still ~14% above that estimated by the Cavalieri Principle.

**DISCUSSION**

The lung poses unique problems for morphometric studies in that it should be preserved at a physiologically relevant state of inflation. To achieve this, it is commonly fixed under a standardized positive airway pressure, which is maintained for several days to weeks until the tissue is presumably stabilized or stiffened. Eventually, the organ must be cut into slices and tissue blocks; at this time, any positive distending pressure that could keep soft tissue parts under tension is eliminated so that septa will assume a relaxed shape. This is the state of lung expansion that will be reflected in the tissue samples viewed in the microscope.

Although pressure dependence of lung volume is widely recognized in vivo, the large pressure-related volume change that can occur after fixation is not as well appreciated. We show here that with tracheal instillation of fixatives, the volume of the intact fixed lung under positive pressure is systematically higher by 13–25% than that measured after sectioning and release of airway pressure. The difference reflects two possible processes: 1) residual elastic recoil was not abolished by fixation and 2) folding or crumpling of fixed alveolar septa.

Oldmixon et al. (11) investigated why alveolar septal profiles appear wavy in dog lungs fixed by perfusion of glutaraldehyde at high airway pressures and concluded that elastin is not fully stiffened until after the lung is dehydrated by ethanol perfusion while inflated. They directly demonstrated that glutaraldehyde fixation alone does not eliminate tissue compliance. Estimated airspace dimension is 30–40% lower when airway pressure is released before the ethanol dehydration step compared with that when pressure is released after dehydration. After ethanol dehydration, the septal profiles appeared straightened out. Bachofen et al. (1) also pointed out that perfusion fixation in excised rabbit lungs using glutaraldehyde does not eliminate tissue recoil forces, leading to an undetermined amount of lung retraction with release of transpulmonary pressure. Mazzone et al. (8) reported ~15% shrinkage in isolated dog lung fixed by perfusion with glutaraldehyde at a transpulmonary pressure of 25 cmH$_2$O; the extent of shrinkage is similar to that after freeze substitution using 70% ethylene glycol with and without fixatives present. The shrinkage of dog lung during air-drying fixation is ~36% associated with moderate shape change (10).

In earlier morphometric studies, the routine procedure was to allow pressure equilibration by cutting the airway and allowing the fixative to escape while the lung floats on a fluid bath before measuring lung volume by fluid displacement. This method requires some care to avoid fluid entry into the lung or uncontrolled fluid escape by squeezing. It is unclear how much fluid should be allowed to escape and how the amount of residual fluid within the lung during immersion measurement could be standardized. The weight of residual fluid exerts a hydrostatic pressure on the dependent part of the lung even after airway pressure is released. We reasoned that this hydrostatic pressure may cause an overestimation of lung volume by immersion, and the effect should be greater in large dog lungs than in small rodent lungs. These predictions are confirmed by the present findings.

Even though we took care to maintain airway pressure while separating the lobes, it is likely that some fixative may have escaped during the procedure causing airway pressure and lung volume to decrease. Lung volume may also have decreased if small leaks developed during storage before volume measurement. Such small leaks may be responsible for the large intragroup variability in $V_{cav}/V_{imm}$ ratio in dog lungs. Any leakage would have reduced the volume measured by immersion and minimized the difference between the two methods. Hence, our reported 25% volume reduction from immersion-to-Cavalieri methods represents a conservative lower estimate of potential shrinkage.

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**Table 1. Volume ratio estimated at time of lung sectioning**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Animals</th>
<th>Volume Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cavalieri/Immersion</td>
</tr>
<tr>
<td>Dog sham</td>
<td>44</td>
<td>0.75 ± 0.18</td>
</tr>
<tr>
<td>Upper stratum</td>
<td></td>
<td>0.65 ± 0.17</td>
</tr>
<tr>
<td>Lower stratum</td>
<td></td>
<td>0.79 ± 0.16</td>
</tr>
<tr>
<td>Dog pneumonectomy</td>
<td>30</td>
<td>0.74 ± 0.13</td>
</tr>
<tr>
<td>Upper stratum</td>
<td></td>
<td>0.71 ± 0.15</td>
</tr>
<tr>
<td>Lower stratum</td>
<td></td>
<td>0.76 ± 0.09</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>63</td>
<td>0.87 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SD. *$P < 0.0003$ guinea pigs vs. dogs by ANOVA. †$P < 0.0001$, ‡$P < 0.02$ upper vs. lower stratum by paired t-test.
variable airway pressure at the time of immersion measurement would have increased the variability of our estimated volume ratios. Airway pressure in the fixed lung could also decrease as trapped air bubbles dissolve or slowly equilibrate across the pleural surface, causing immersion volume to decrease with time, although these factors are minor as our serial data show a less than 10% progressive volume loss over 3 mo. It is possible that while the lung is immersed in fixatives, the tissue (cells and collagen matrix) may become sufficiently stiffened with time so as to withstand the retractive force of extended elastin to some degree. However, this process is incomplete as evidenced by the “wavy” appearance of septal profiles on sections.

The major advantage of the Cavalieri Principle is that it allows an estimate of the total volume of the organ and, in the case of the fluid-filled lung, in a state of relaxation free of residual tissue elasticity. Michel and Cruz-Orive (9) reported an excellent agreement between volume estimation by the Cavalieri and fluid displacement methods in small rabbit lungs (~50 ml) where the measurement of lung volume by immersion was done after release of pressure. Pache et al. (12) applied the Cavalieri Principle to measure lobar lung volumes from computerized tomographic images of an infant lung (total volume 45 ml, each lobe 5 to 15 ml) and found that lobar volumes agreed closely with that measured by immersion after release of airway pressure. On the other hand, we found a persistent 14% difference in dog lungs (volume 1,000–1,500 ml/lung or 500–800 ml/stratum) even after release of airway pressure, suggesting that the two methods do not correspond as well for large lungs.

The systematically lower $V_{\text{cav}}/V_{\text{imm}}$ of the upper stratum in dog lung is unexpected but consistent with in vivo regional differences in lung mechanics. The upper lobe has been shown to contain significantly more air per gram of lung tissue than did the lower lobes at a given transpulmonary pressure in the dog (2), monkey (13), and human (6). In the in vivo dog lung (14) and excised human lung (15), compliance of the upper lobe is higher than in the lower lobe of the same lung. The difference has been attributed to regional differences in surface forces and/or tissue elasticity in the air-filled lung. Because surface forces are eliminated in the fluid-filled lung after fixation, the observed regional difference must be attributed to tissue elasticity. However, the anatomic explanation is not fully understood.

These results highlight an important source of variability in lung volume measurement after fixation by the immersion method, in addition to the shrinkage that occurs during subsequent histological processing (7). These data emphasize that for morphometric analysis, the optimal measurement of lung volume should be one obtained as close to the sampling and embedding steps as possible. Volume measured after sectioning by the Cavalieri method is therefore preferable to that measured by saline immersion even after complete release of airway pressure, particularly for large lungs.

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