TITLE:
Lung Fixation under Constant Pressure for Evaluation of Emphysema in Mice

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KEYWORDS:
dhronic obstructive pulmonary disease, emphysema, lung fixation, constant pressure, emphysematous mouse model, cigarette smoke

SUMMARY:
Presented here is a useful protocol for lung fixation that creates a stable condition for histological evaluate of lung specimens from a mouse model of emphysema. The main advantage of this model is that it can fix many lungs with the same constant pressure without lung collapse or deflation.

ABSTRACT:
Emphysema is a significant feature of chronic obstructive pulmonary disease (COPD). Studies involving an emphysematous mouse model require optimal lung fixation to produce reliable histological specimens of the lung. Due to the nature of the lung’s structural composition, which consists largely of air and tissue, there is a risk that it collapses or deflates during the fixation process. Various lung fixation methods exist, each of which has its own advantages and disadvantages. The lung fixation method presented here utilizes constant pressure to enable optimal tissue evaluation for studies using an emphysematous mouse lung model. The main
advantage is that it can fix many lungs with the same condition at one time. Lung specimens are obtained from chronic cigarette smoke-exposed mice. Lung fixation is performed using specialized equipment that enables the production of constant pressure. This constant pressure maintains the lung in a reasonably inflated state. Thus, this method generates a histological specimen of the lung that is suitable to evaluate cigarette smoke-induced mild emphysema.

INTRODUCTION:

COPD is one of the leading worldwide causes of death\(^1\). Cigarette smoke is the most important cause of COPD, but the mechanisms of pathogenesis remain incompletely defined. COPD demonstrates two main characteristics, including progressive limitation of airflow and an abnormal inflammatory response of the lung. Emphysematous disorder frequently occurs in the lungs of COPD patients\(^2\). The pathological findings of emphysema are characterized by alveolar wall destruction\(^3\). Several animal species have been used to generate COPD models in vivo (i.e., dogs, guinea pigs, monkeys, and rodents)\(^4\). However, the mouse has become the most commonly used in the construction of COPD models. This has many advantages, including its low cost, ability to be genetically modified, extensive genomic information availability, availability of antibodies, and ability to use a variety of mouse strains\(^5\). Presently, there is no mouse model that can mimic the full features of human COPD; thus, individual researchers must choose which model is most suitable for the specific COPD research\(^6\). The emphysematous mouse model is one of many COPD mouse models that are currently available. Additional models include the exacerbation mouse model, systemic co-morbidities model, and COPD susceptibility model\(^7\).

The emphysematous mouse model can be generated by several types of exogenous agents, including chemical agents and cigarette smoke exposure\(^8\). Chemical exposure (e.g., to elastase) produces a severe type of emphysema, while cigarette smoke results in mild emphysema\(^8,9\). Cigarette smoke is believed to be the main cause for the pathogenesis of COPD; therefore, the choice of cigarette smoke as a means to create a COPD mouse model is reasonable\(^10\). Many studies have used cigarette smoke to create emphysema in the mouse. For example, Nikula et al. successfully created an emphysematous mouse model from B6C3F1 female mice by exposing them to cigarette smoke for 7 or 13 months\(^11\). We have also established an emphysematous mouse model via senescence marker protein/SMP-30 KO mice\(^12\). It is crucial to perform a lung fixation method that can properly visualize this mild emphysema model by cigarette smoke exposure.

Various methods for lung fixation have been established\(^13\). However, there is no gold standard method of lung tissue fixation for evaluating emphysema\(^14\). Several studies from this lab have shown that the fixation system presented here is useful by creating a stable condition for evaluating emphysema\(^12,15-18\). The main advantage of the current system is that it can fix many lungs with the same condition at one time without lung collapse or deflation. The current lung fixation system uses some special equipment that allows lung specimens to be inflated at an appropriate constant pressure for a given period. This special equipment consists of three parts, including a lower container, upper container, and pump. Lung specimens are placed in
the lower container that is connected to pressurized fixing agents, resulting in a 25 cmH$_2$O pressure difference in the level of agents between the upper and lower containers $^{19}$. 

**PROTOCOL:**

The following methods have been approved by the Animal Care and Use Committees of Juntendo University School of Medicine. There are three main steps in this method: 1) mouse dissection, 2) lung exsanguination, and 3) fixation of lung tissues assisted by specialized equipment. Typically, lung specimens are processed to embedment after 48 h of fixation $^{12,15-18}$.

**1. Mouse dissection**

1.1. Measure the body weight of the mouse, then determine the amount of pentobarbital to administer.

1.2. Inject pentobarbital intraperitoneally at a dosage of 70 mg/kg of body weight and confirm anesthesia by the absence of reaction to toe pinch.

1.3. Inject the needle at a 45° angle until it penetrates the skin and muscle. Draw the plunger and confirm an air vacuum, then inject the pentobarbital.

1.4. Confirm anesthesia by the absence of reflex motion.

1.5. Cut the mouse skin and abdominal muscle at the medial line, aiming for the cephalic area.

1.6. Cut laterally to provide a wider working space.

**2. Lung exsanguination**

2.1. Expose the diaphragm layer and puncture it with forceps.

2.2. Open the thoracic space and cut the sternal area, allowing the lungs and heart to be seen clearly.

2.3. Cut the heart in four locations: right atrium, left atrium, left ventricle, and right ventricle.

2.4. Insert a cannula (22 G) into the right ventricle area and direct it to the cephalic area until it reaches the pulmonary artery, as shown in **Figure 1**.

2.5. Turn on the pump and allow the 1x phosphate-buffered saline (PBS) to circulate (approximately 200 mL/h) until all lung tissue changes to a white color.

**3. Fixation of lung tissue**
3.1. Remove the trachea, lungs, and heart.

3.2. Free all three organs by cutting the surrounding connective tissues.

3.3. Tie the right main bronchus with a suture thread and cut all lobes of the right lung.

3.4. (Optional): the lobes of the right lung consist of four parts. Cut these parts from the right main bronchus and divide the parts for processing as frozen tissue samples.

3.5. Insert the heart and the lobes of the left lung into fixing agents, located inside a 10 mL syringe.

CAUTION: Fixing agents are hazardous. Wear proper protective equipment (e.g., long rubber gloves) and work in a well-ventilated room.

3.6. Create a vacuum condition using a 10 mL syringe to inflate the lung, as shown in Figure 2.

3.7. Insert a cannula (20 G) into the trachea and tie a knot.

3.8. Inflate the lung with fixing agents to check with for leaks, using a 1 mL syringe.

3.9. Transfer to lung fixation pressure equipment, as illustrated in Figure 3.

3.10. After fixing periods, remove the lung sample tying the trachea off with a knot.

REPRESENTATIVE RESULTS:

As described previously, the specialized equipment, which generates extended constant pressure, can be divided into three parts (Figure 3A). The lower part is the point at which to insert the lung sample (Figure 4A). The lung is connected via a cannula (20 G) to the tip of formalin flow using a three-way stop cock (Figure 4B). Pressure is generated from the different surface levels of fixing agents between the lower and upper containers (Figure 5). The pressure difference is 25 cmH₂O; however, using the height adjustment knob, the pressure can be adjusted within the range of 25–30 cmH₂O (Figure 5). A pump connects the lower and upper containers via tubes (Figure 3A), preserving a 25 cm difference in fixing agent surface height. The direction of agent flow is described in Figure 3B.

Presented next is a representative result of histological findings in the lung, following 48 h of fixation. Six-month-old male SMP30-KO mice were exposed to cigarette smoke or fresh air (as control) for 8 weeks. Both tissue specimens were stained with hematoxylin and eosin. Figure 6A shows histological findings from the air-exposed mice, which did not exhibit marked airspace enlargement. In contrast, Figure 6B reveals significant airspace enlargement and alveolar wall destruction in mice that were exposed to chronic cigarette smoke.
The mean linear intercepts (MLI) was determined according to the method described by Thurlbeck et al.\textsuperscript{20} to access the airspace size. The destructive index (DI) was determined to evaluate the destruction of the alveolar wall according to the method described by Saetta et al.\textsuperscript{21}. These morphometric examinations of the lung specimen revealed that DI and MLI were significantly greater in the smoke-exposed SMP30-KO mice than in the air-exposed mice (Figure 6C,D).

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Lung exsanguination.** A cannula was inserted at the location of the right ventricle and directed to the pulmonary artery.

**Figure 2: Vacuum syringe lung inflation.** Vacuum condition inside the 10 mL syringe containing fixing agents to inflate the lungs.

**Figure 3: Lung fixation equipment.** (A) The acrylic equipment allowed a 25 cmH\textsubscript{2}O pressure difference to inflate the lungs continuously for 48 h, utilizing a pump machine. (B) The direction of fixing agent flow is indicated by arrows.

**Figure 4: Lower container.** (A) The mouse lung specimen was positioned inside fixing agents in the lower container. (B) Inside the lower container, there is a sample placement box, at the top of which formalin flows through a three-way stopcock and the cannula.

**Figure 5: Upper container and height adjustment knob.** The upper container generated a pressure of 25 cmH\textsubscript{2}O. There are two pairs of height adjustment knobs which can be used to adjust the height of the upper container; as a result, the pressure that is generated can be set within the range of 25–30 cmH\textsubscript{2}O.

**Figure 6: Mouse lung histologic and morphometric findings.** Representative histologic images of lung sections from 8-week cigarette smoke-exposed or air-exposed SMP30-KO mice (6 month-old, male), stained with hematoxylin-eosin. Scale bar = 100 \textmu m. (A) The air-exposed group did not show significant enlargement or other findings. (B) The cigarette smoke-exposed group showed marked airspace enlargement and alveolar wall destruction. (C) The mean linear intercepts (MLI). In the lungs of cigarette smoke-exposed mice, MLI was significantly greater than air-exposed mice (*p < 0.001). (D) The destructive index (DI). In the lungs of cigarette smoke-exposed mice, DI was significantly increased compared to the lungs of air-exposed mice (*p < 0.001). Values are presented as mean ± SD (n = 6 for each group).

**DISCUSSION:**

The fixation procedure for rodent lungs presented here is not novel; however, this system has several advantages. Firstly, it can fix many lungs (maximum of 20) with the same condition at one time. The Society of Toxicologic Pathology states that the pressure for gravity instillation vary from 22–25 cmH\textsubscript{2}O.\textsuperscript{22} Notably, several studies have performed lung fixation at a pressure...
of 25 cmH₂O^{13,19,23-27}, which has been adopted in our laboratory using the current system^{12,15-18}. Secondly, it can fix lung tissues at a constant pressure for various periods of time. In our laboratory, lung samples are usually fixed for 48 h. Many investigators use a relatively short period of time (e.g., 5–20 min)^{13,28-32}, then tie off the inflated lung and immerse in formalin for extended periods as desired. There is no data or research indicating a gold standard for the length of duration for lung fixation. However, the statement of the American Thoracic Society (ATS)/European Respiratory Society (ERS) describe the “silver standard”, in which airway inflation pressure must be maintained for at least 24 h^{24}. The Japanese Society of Pathology also recommended fixation times of no longer than 1 week to produce consistent immunohistochemical slides; although, their recommendation is based on analysis using human specimens^{33}. Relatively short fixing time periods may not be applicable to the current system, because each sample is supposed to be individually placed in the lower container. This is a limitation of the current system. In conclusion, the proper length of time for mouse lung fixation remains unknown.

Critical steps in this method are related to the risk of lung formalin leakage during the formalin fixation process. Lung formalin leakage can cause lung size shrinkage. This risk can be divided into two parts. The first part occurs during the sacrifice step. While opening the thoracic cage, it is important not to cause injury to the lung surface. The key to prevention is to approach this from the diaphragm and continue to cut the thoracic rib cage after the lung is detached from the parietal pleura. This method avoids lung injury caused by surgical equipment. Another key step occurs when tying the right main bronchus. It is important to identify which are the mouse’s right lobes. Placing the lungs in a position where they can be seen from a dorsal view enables easier identification of the location of the lungs.

The second part is during the lung fixation process using specialized equipment. A critical step occurs while inserting the lung specimen into the lower container’s formalin port. It should be confirmed that the insertion is tightly secured to prevent lung specimen detachment from the formalin port during the constant pressurization process. Another aspect to highlight is the tubing connection between the three parts of specialized equipment (lower container, upper container, and pump). All tube connections should be tightly connected. If leakage occurs, the formalin volume in the upper container will decrease, thereby reducing constant pressure.

According to recommendations from the Society of Toxicologic Pathology, intratracheal instillation of formalin has advantages for rodent lung model, which prevail over its disadvantages^{22}. They have suggested the use of an intratracheal formalin fixation method when performing quantitative studies of alveolar lung morphometry. Intratracheal lung instillation has two advantages, including preservation of the airway and alveolar wall as well as visualization of lung parenchyma^{22}. One study by Braber et al. revealed that the intratracheal formalin instillation method is superior in terms of preserving lung structure when compared with the vacuum inflation and whole-body perfusion methods^{13}. The current method utilizes intratracheal instillation in a mouse model to optimize visualization of the alveolar area.
Regarding fixing agents, 10% formalin, which contains formaldehyde, is conventionally used. Formaldehyde is widely used as a fixing agent for immunopathological investigations because it does not completely destroy protein immunogenicity. However, the ATS/ERS statement does not recommend formalin fixation, because it does not adequately stabilize tissue structure. Glutaraldehyde is recommended for airway instillation instead; however, it is subject to destroy protein immunogenicity, which results in an unsuitable fixing agent for immunohistochemistry. Several pieces of evidence have reported that the fixed lungs may be provided for morphometric evaluation (e.g., mean linear intercepts, internal surface area, and destructive index) following formalin fixation using the current fixation system. Certainly, glutaraldehyde can be adopted for the current system; thus, researchers can choose both agents in the current system according to experimental needs.

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DISCLOSURES:
The authors have no competing interests to declare.

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Index finger closed the opening

Inflated lung tissue

Withdrawing the plunge
A. Lung fixation equipment

B. The direction of fixing agent flow
A. Lower container

![Sample placement box and three-way stopcocks]

B. Sample placement box and three-way stopcocks
A. Air-exposed lung

B. Cigarette smoke-exposed lung