

Chapter 2

The Bleomycin Model of Pulmonary Fibrosis

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Abstract

Interstitial lung disease (ILD) comprises a large number of chronic lung disease characterized by varying degrees of inflammation and fibrosis. Mostly they are idiopathic including idiopathic pulmonary fibrosis (IPF), which is a specific disorder characterized by progressive fibrosis leading commonly to end-stage lung disease, respiratory failure, and fatal outcome. IPF and many of these fibrotic ILDs lack effective therapy despite recent approval of two drugs to slow progression in certain IPF patients. Because there are no natural models for IPF, the use of animal models that reproduce key known features of the disease is warranted. Thus, different animal models have been developed to investigate key mechanisms underlying pathogenesis of pulmonary fibrosis and identify potential therapeutic targets for IPF. While no animal model can recapitulate all features of human disease, several are available to address select features of IPF and other fibrotic ILDs. Historically, among the first to be developed and used widely is the bleomycin model, which is the best-characterized and currently most extensively used animal model due to its ability to reproduce many aspects of IPF and other fibrotic ILDs, good reproducibility, and ease of induction. Studies using the bleomycin model have identified many of the cellular and molecular mechanisms now recognized as being important in pathogenesis of IPF and other fibrotic ILDs, as well as novel therapies for these diseases, including two recent drugs approved for treatment of IPF. This chapter will describe commonly used techniques for induction of the model by endotracheal administration of bleomycin through surgical and nonsurgical (transoral instillation).

Key words Bleomycin, Idiopathic pulmonary fibrosis, Mouse, Endotracheal instillation

1 Introduction

1.1 General Considerations

Chronic fibrosis is often associated with many ILDs, and for many in the idiopathic category, there is currently no effective therapy due in part to lack of known etiology and adequate knowledge of their natural history and/or pathogenic mechanisms. In IPF this is characterized by alveolar epithelial cell damage, subsequent release of pro-inflammatory and pro-fibrotic cytokines and other mediators, accumulation of activated fibroblasts and myofibroblasts in the fibrotic foci, and the excessive abnormal deposition of extracellular matrix proteins. Inexorable progression of fibrosis results in loss of normal lung architecture, end-stage lung disease,

respiratory failure, and eventual fatal outcome [1–3]. IPF is characterized by the histological pattern of usual interstitial pneumonia [4]. Although no single animal model of pulmonary fibrosis recapitulates all features of the human disease, use of animal models has led to reproduction of many of the manifestations of IPF. Among the various animal models of pulmonary fibrosis (bleomycin, FITC, silica, radiation, etc.), the bleomycin model is the most extensively used and best-characterized murine model in use today [5, 6].

Bleomycin is a family of complex glycopeptides [7] with antineoplastic properties, initially isolated from a strain of actinobacteria, *Streptomyces verticillus* [8]. Its primary clinical use is as an antitumor antibiotic for various carcinomas and lymphomas [9–11]. Bleomycin-induced toxicity occurs predominantly in the organs of the lung, skin, and mucous membranes due to the lack of the bleomycin-inactivating enzyme, bleomycin hydrolase in those tissues [9, 12, 13]. Intradermal injections of bleomycin can be used to exclusively model skin fibrosis (method detailed in Chap. 3). Pulmonary fibrosis is a well-known side effect of bleomycin, because the lung expresses very low levels of this enzyme. This enables persistence of the compound with consequent greater susceptibility to bleomycin-induced lung injury relative to other organs [13–17]. The use of bleomycin is limited by the development of pulmonary fibrosis in 3–5% of patients receiving this chemotherapeutic agent [5]. This notable and undesirable side effect of bleomycin leads to its use in animal models studying mechanisms of pulmonary fibrosis of relevance to IPF. Bleomycin as an agent to induce experimental lung fibrosis is first described in dogs and subsequently in mice, hamsters, rats, and sheep [18–22]. Eventually, it becomes an established model in rodents for the study of pathogenic mechanisms involved in both acute and chronic stages of fibrotic lung diseases and for identification of novel therapeutic targets followed by evaluation of relevant potential therapies [21, 23].

Delivery of bleomycin to the lung causes pulmonary injury, inflammation, and subsequent fibrosis [5]. In the presence of iron and oxygen, bleomycin can generate reactive oxygen species (ROS) and cause DNA strand scission, which is thought to be the basis for its antitumor activity and presumed mechanism for causing cytotoxicity and tissue injury [5, 24, 25]. However, the doses used in the rodent model of fibrosis appear not to cause significant DNA damage [26], thus making it unclear if this is the basis for the injury in this model. However the ROS produced in response to bleomycin can also cause lipid peroxidation and protein oxidation, which may be involved in the mechanism driving fibrosis in this model [5].

1.2 Advantages and Disadvantages of the Bleomycin Model

Due to its early historical introduction and thus more extensive experience with it, the bleomycin model is best characterized in many important aspects of pulmonary fibrosis with limited relevance to IPF. These include in vivo mechanisms of TGF- β

activation, epithelial cell injury and basement membrane damage, and interstitial and intra-alveolar fibrosis with dense collagen deposition [27–29]. The model has provided useful information encompassing both acute and chronic lung injury along with the reparative stage of the fibrotic process. Some of the molecular signatures as well as some histopathological hallmarks at distinct stages of bleomycin-induced lung fibrosis resemble those encountered in human fibrotic lung diseases, including IPF. In this regard, it is noteworthy that the preclinical development of two drugs, pirfenidone and nintedanib, recently approved for slowing progression of certain subsets of IPF patients, has been done using the bleomycin model [30–32]. Moreover with respect to pirfenidone, its effectiveness was first discovered using the bleomycin model [33]. The acute injury stage of the response to bleomycin has also been exploited as a model of acute lung injury, such as in acute respiratory distress syndrome (ARDS) as well as potential utility as a model of acute exacerbation in IPF [34, 35]. Thus in addition to its utility as a model of chronic fibrotic lung disease, it is useful as well for modeling more acute stages of lung injury that can lead to fibrosis. The murine model is also particularly useful for elucidation of the role of genetic background in influencing susceptibility to IPF and other fibrotic ILDs because of the strain differences in responsiveness to bleomycin-induced pulmonary fibrosis [36–38]. Recent studies have also exploited the model for studying the role of aging in increased susceptibility to IPF [39]. Thus, despite its imperfections as a model of human chronic progressive lung disease, the bleomycin model has been quite useful for elucidating mechanistic insight and drug discovery, perhaps in a more limited fashion. It has certainly been useful for evaluation of the *in vivo* fibrogenic significance of descriptive observations emanating from clinical studies of IPF and other fibrotic ILDs.

A key additional advantage of the bleomycin model of pulmonary fibrosis is the ease of induction depending on the method chosen and its good reproducibility. It can be delivered locally or systemically, including endotracheal, intranasal, intravenous, and intraperitoneal routes. Using the local approach, administration of a single dose of bleomycin produces a rapid development of injury and acute inflammation followed by a chronic inflammatory phase with the development of fibrosis over the subsequent weeks. More severe fibrosis is noted from repetitive drug dosing; however, key molecular signatures are similar between single vs. repetitive dosing [40–42]. Depending on the dose of bleomycin and/or the murine strain used, this time course of events and intensity of injury or fibrosis can vary significantly, thus enabling modeling of select stages of disease (e.g., early vs. late, acute exacerbation vs. normal progression, etc.) and/or type of disease (e.g., ARDS vs. IPF). A significant advantage is that the development of fibrosis is relatively quick with the peak fibrotic response occurring at around

3–4 weeks after a single dose of drug via endotracheal delivery. The shorter time frame and reproducibility also mean reduced costs associated with animal purchase and husbandry. Thus, in addition to being economical (especially the single-dose model), the strengths of the bleomycin model lie in its relative ease of induction, reproducibility, and versatility [43].

A key criticism, hence disadvantage, of the bleomycin model is the self-limiting nature of the fibrosis, which contrasts with the progressive chronic fibrosis typical of the known natural history of human IPF [6, 44, 45]. There is a suggestion that repetitive bleomycin dosing may ameliorate some of this criticism with respect to modeling of human IPF [40]. The claim of spontaneous resolution of fibrosis in the bleomycin model represents another potential disadvantage since the unending progression of fibrosis in IPF usually ends in end-stage lung with honeycombing and certainly no evidence of spontaneous resolution of fibrosis [44, 46]. However, this is not universally observed since there are reports showing the persistence of fibrosis in the bleomycin model up to 3–6 months [6, 47]. The basis for this discrepancy is unclear but may be related to the dosing of bleomycin and/or murine strain used in the different studies. Another common criticism is the role of inflammation in the model. As noted in the time course of events after bleomycin treatment, significant chronic inflammation is evident and appears to be important for the developing fibrosis, which appears not to be the case in IPF based on lack of responsiveness to anti-inflammatory steroid treatment and dearth of inflammatory cells in IPF lung tissue [44, 45, 48]. Some studies however indicate only limited, if any, dependence of fibrosis on the inflammation noted in the model [49]. A way to demonstrate this has been to separate the fibrosis from the inflammation by examining the effects of interventions during the fibrotic phase of the model (usually days 14–28 after model induction) when the inflammation is starting to wane [50]. Recent studies have attempted to tweak the model in such a way as to highlight certain more limited phases of fibrogenesis observed in IPF. These modifications or modified use of the model have reduced but not eliminated many of the noted limitations. Nevertheless despite these limitations, the many significant advantages of the model and its track record for elucidating in vivo mechanisms plus utility for drug discovery make the bleomycin model a valuable asset for preclinical and mechanistic studies. It certainly remains as one of the most widely used model for studies of chronic progressive lung diseases today and arguably the best available experimental model in this regard.

1.3 Model Progression

The development or progression of lung injury/fibrosis in various bleomycin animal models is quite similar regardless of species and routes of administration. The lung injury progresses through three stages. (1) Acute injury and inflammatory phase (1–7 days

post-endotracheal injection of bleomycin) are characterized by influx of inflammatory cells with activation and elaboration of a multitude of inflammatory mediators due to widespread damage to the epithelium, combined with vascular leak, upregulation of pro-inflammatory cytokines, and chemokines [6]. (2) Transition phase from inflammation to active fibrosis (7–14 days post of bleomycin) shows gradual subsidence of the inflammatory response with accompanying increase in fibroproliferation, initiation of myofibroblast emergence, and interstitial collagen gene expression peaking toward the end of this phase and beginning of the next [50]. (3) After the third week post-bleomycin is the chronic fibrosis stage when intra-alveolar and septal fibrosis becomes morphologically evident. This phase is characterized by the expansion of the myofibroblast population and increased deposition of extracellular matrix. Lung collagen synthesis remains elevated along with production of other extracellular matrix components as part of the attempted repair process. The increased deposition of lung collagen usually peaks at about 28 days post-bleomycin treatment [6, 51–53]. The development of fibrosis in this model can be assessed histologically and biochemically by measurement of lung hydroxyproline content to estimate total lung collagen content. Some reports show that the fibrotic lesions resolve after day 21–28 [6, 45], while other recent studies indicate persistence of fibrosis, albeit with less inflammation as long as 6 months after a single or repetitive bleomycin treatment(s) [40, 47]. Moreover, these studies show that the bleomycin model has many similarities with human IPF. Despite this discrepancy regarding resolution of fibrosis, there is agreement with respect to the lack of progression beyond the first month after bleomycin treatment, namely, active fibrosis wanes, and no further increase in fibrotic lesions are noted. Figure 1 shows the characteristic pathology of lung fibrosis caused by a single dose of bleomycin through transoral instillation. Fibrosis is fully developed with extensive and diffuse involvement of the lung at the third week (Fig. 1b), with persistence of the developed fibrotic lesions at 8 (Fig. 1c) and 12 (Fig. 1d) weeks after bleomycin instillation. However no progression or extension of fibrotic lesion areas is noted beyond the third week. Repetitive dosing with bleomycin results in similar fibrotic outcome without significant differences in key fibrotic parameters [40].

1.4 Experimental Design

The detailed protocols in this chapter are designed for the bleomycin mouse model induced by endotracheal administration via non-surgical transoral instillation or direct injection into the trachea. The approach can also be modified for the repetitive model by adjusting to a lower dose of bleomycin (e.g., 50% of the single instillation dose) using the transoral route of administration at the desired frequency and intervals in between treatments. It can also be adapted for the rat model by increasing the drug dose/volume, adjusted accordingly for this species and based on the body weight.

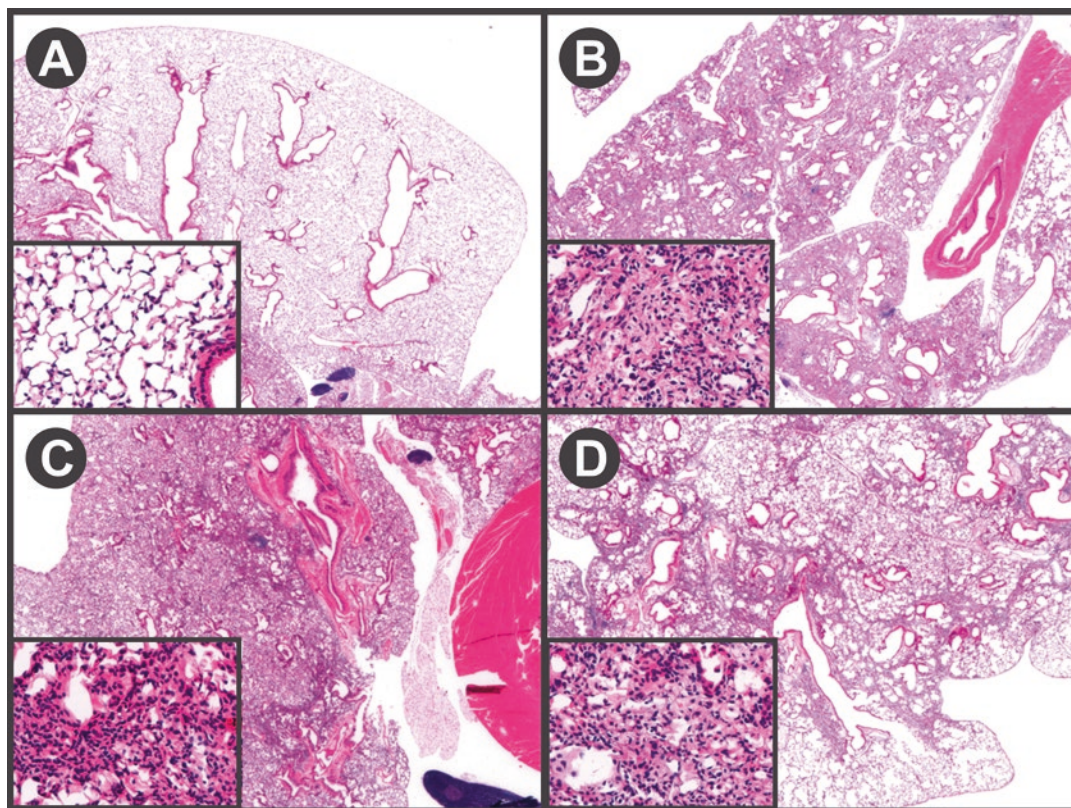


Fig. 1 Lung histopathology after single dose of bleomycin treatment. Bleomycin (2 U/kg) was administered into an 8-week-old female C57BL/6 mice by transoral instillation at day 0. Representative hematoxylin- and eosin-stained lung tissue sections are shown. The mice were sacrificed at 3 (**a** and **b**), 6 (**c**), and 12 (**d**) weeks after bleomycin instillation. Except for the PBS control (**a**), all panels showed lung section from bleomycin-treated mice. The lungs were inflated with 10% buffered formaldehyde. Original magnification, 20 \times ; insets: 200 \times

Several factors should be considered before selecting the appropriate bleomycin model to use, including the route for delivery, dose, dosing frequency, animal gender, and genetic or strain background.

1.4.1 Route of Administration

Although recent findings suggest that repetitive endotracheal administration of bleomycin mimics the chronic aspect of pulmonary fibrosis [54, 55], other studies show little difference between the repetitive dosing vs. single endotracheal dosing with respect to the development of fibrosis [41, 47]. The method of delivery is similar although the dosing per treatment in the repetitive model is reduced [40]. A significant disadvantage of the repetitive model is the greater length of time needed for fibrosis to be fully developed, and thus the increased cost of both bleomycin and animal husbandry. In the more widely used model, bleomycin is usually given by a single dose in weight-adjusted dosages. The routes of drug delivery include direct endotracheal injection or via transoral

instillation and systemically via intravenous or intraperitoneal injections. The direct endotracheal delivery of bleomycin is more widely used than systemic approaches because of relative ease of induction and is more economical since lower and less frequent dosing is required, plus the shorter time course of the study. Systemic administration is thought to cause more diffuse disease with a slower time course for development of fibrosis, which may be more representative of events in IPF and also may be useful to mimic the usual delivery route in clinical application as a chemotherapeutic agent [46]. A significant disadvantage is the length of time needed and the requirement for higher and repeated doses of bleomycin. Traditional endotracheal delivery of bleomycin is performed by a surgical procedure. Trachea of the mouse under anesthesia is exposed by surgical incision, and bleomycin is then directly injected into the trachea [44, 56]. This endotracheal delivery method has since been modified by use of a transoral approach and is now more widely used [5, 47, 57]. Transoral instillation is quicker and noninvasive since no surgery is required, thus allowing more rapid recovery, and postsurgical monitoring is eliminated. It may also produce a more uniform distribution due to gravity and natural inhalation by the mouse [5, 48]. The protocol described in this chapter is focused on this transoral instillation approach for its ease, efficient, and reproducible use.

1.4.2 Genetic and Gender Variation

The role of gender in animal model studies of pulmonary fibrosis is controversial. In rats, females exhibit greater sensitivity to bleomycin with higher levels of fibrosis compared to males, which is abolished by ovariectomy [58]. Moreover estrogen treatment of male rats enhances their response to bleomycin. In contrast studies in mice reveal either no significant difference or a greater male responsiveness in bleomycin-induced pulmonary fibrosis, although males exhibit greater decline in lung function [5]. The genetic susceptibility to bleomycin is a heritable trait controlled by a few genetic loci, and X-linked factor may be associated fibrosis phenotype [38]. The susceptibility to bleomycin in mice is strain and genetic background dependent with C57BL/6 mice being high responders and DBA/2 mice being intermediate, while BALB/c mice are relatively bleomycin resistant [52]. The basis for the noted differences is not entirely clear but is likely due to different expression levels of bleomycin hydrolase, as well as expression patterns of cytokines and proteases/antiproteases [36–38].

1.4.3 Dosage of Bleomycin

A wide range of doses of bleomycin for model induction is reported in the literature. In part this could be due to the fact that the potency of bleomycin may vary between companies and their lot numbers. The response to the drug is also mouse strain dependent. Thus, an optimal dose for the desired effect has to be determined in preliminary studies for each batch of bleomycin. The commonly used doses

through direct endotracheal administration into the lung are CBA/J mice, 1 U/kg; C57BL/6, 2 U/kg; BALB/c, 10 U/kg; and Fisher 344 rat, 7.5 U/kg [56, 57, 59]. Mortality is usually minimal at these doses, while increasing the dose can significantly increase mortality in most of these animals, often during the acute injury phase and before chronic fibrosis is fully developed. For systemic administration the dose is generally 10–20 times higher, while in repetitive dosing, the dose is usually half the single-dose protocol.

1.4.4 Selection of Time Point for Analysis

Choosing the appropriate endpoint in the bleomycin model depends on the specific parameter of the injury and fibrotic process that needs to be evaluated. Analyses during the first 3 days will reflect the acute injury and inflammatory response to bleomycin. Bronchoalveolar lavage fluid (BALF) is often analyzed for changes in total and differential cell counts. The fibroproliferative response manifested by increasing numbers of fibroblasts and myofibroblasts usually occurs 2 weeks after local delivery of bleomycin, so this is an appropriate time point to study mesenchymal cell proliferation. To evaluate established fibrosis in lung tissue, 3–4 weeks after drug treatment should be considered because the peak of collagen deposition appears at this time point [5]. Semiquantitative histological analysis, quantification of total collagen content by hydroxyproline analysis, as well as the assessment of lung myofibroblast differentiation as measured by α -smooth muscle actin expression may be conducted during this period [5, 60].

1.4.5 Therapeutic Intervention

The bleomycin model has been extensively used in drug discovery studies for identification of novel therapeutic targets, evaluation of potential efficacy of the compound directed at the identified target, preclinical evaluation, and in proof of concept studies. It is almost routinely used along with another model for demonstration of efficacy. For use in such studies, it is important to distinguish between anti-inflammatory and anti-fibrotic drug effects since the bleomycin model has both acute injury and inflammatory phase with a subsequent fibrotic phase. Thus for evaluation of anti-fibrotic therapy, delayed initiation (e.g., >day 12–14 after bleomycin treatment) of treatment with the test compound should be considered [4, 61, 62]. This approach will obviate effects on inflammation that may make it difficult to distinguish an anti-inflammatory effect resulting in subsequent inhibition of fibrosis vs. a purely anti-fibrotic effect independent of effects on inflammation.

2 Materials

2.1 Transoral Instillation of Bleomycin in Mice

1. Mice, 6–8 weeks old, that have been acclimated for a week in the local facility (*see Note 1*).
2. Phosphate buffer saline (PBS).

3. Bleomycin (we use the one from APP Pharmaceuticals LLC, Schaumburg, IL). Reconstitute bleomycin dry powder with sterile PBS to the desired unit concentration (2–10 U/mL, 1 Unit = 1 mg). Small aliquots of a concentrated stock solution can be stored at -80°C up to a year for future use (*see* **Notes 2** and **3**).
4. Ketamine.
5. Xylazine.
6. 1 mL syringe with 26 $\frac{1}{2}$ G needle.
7. Ophthalmic ointment.
8. Forceps.
9. Heat lamp.
10. Disposable underpads.
11. Surgery board with an approximately 70° angle (from horizontal) with looped surgical suture thread attached at the top (Fig. 2).
12. Scale.
13. Alcohol pre pad.
14. 70% ethanol (v/v, in water).
15. 10 mL syringe with 22 $\frac{1}{2}$ G needles.
16. Paper towels.



Fig. 2 Nonsurgical transoral instillation of bleomycin into mouse lung. The mouse is anesthetized with ketamine/xylazine and suspended on an approximately 70° angled surgery board. Bleomycin is delivered through transoral instillation by using a 200 μL pipet as described in text. An apparatus for the procedure is shown on the *left*, and a view of exposed oral cavity is shown on the *right*

17. 23½ G needles.
18. 15 mL tubes.

2.2 Additional Materials Needed for Direct Endotracheal Injection of Bleomycin

These materials are in addition of the ones listed in Subheading 2.1.

1. Betadine.
2. Sterile scissors.
3. Auto-suture clips 9 mm.
4. Scalpel blades.
5. Electric shaver.
6. Ethanol swabs.

3 Methods

3.1 Transoral Instillation of Bleomycin in Mice

1. Prepare the desire dilution of bleomycin to administer based on body weight.
2. To anesthetize mice using a combination of ketamine/ xylazine: weigh each mouse, and calculate ketamine/xylazine dose per kg body weight (*see Note 4*); restrain the mouse by grasping it by the scruff, and inject 200 µL of ketamine/xylazine solution intraperitoneally (IP) using a 1 mL syringe with 26½ G needle. The duration of anesthesia is 20–30 min.
3. Within 5 min of injection of anesthetic, the mouse will settle down and stop moving. Verify sedation by lack of withdrawal upon firmly pinching a toe, and proceed with the next steps if sedation is achieved.
4. Apply to prevent eyes from drying.
5. Load the required volume of bleomycin or sterile PBS into a sterile 200 µL pipet tip.
6. Place mouse on the surgery board, and suspend via the upper incisors using the surgical thread loop (Fig. 2). Ensure adequate lighting is available for visualization of vocal cords.
7. Pull and extend the tongue gently using sterile padded forceps to one side, toward the mandible to visualize the vocal cords; then, lower the pipet tip loaded with bleomycin into the back of the oral cavity to deliver the liquid through the vocal cords during inspiration (Fig. 2). Wait to hear a gasp, which confirms endotracheal delivery of the liquid. Control animals receive an equal volume of sterile PBS instead of the bleomycin solution.
8. Release the tongue and carefully dislodge the upper incisors from the suspension thread. Place the mouse under a heating lamp or pad until it recovers from the anesthesia, commonly within an hour after injection of anesthetic.

9. Clean the forceps with alcohol pad before and after each use.
10. Monitor the mice daily until they are euthanized for analysis. Body weights and other parameters can be evaluated and recorded daily or as needed.
11. Euthanized the animals at the time points of interest: inject the mice with an overdose of ketamine/xylazine solution based on body weight (1.5 times the dose for regular anesthesia).
12. After 5 min verify sedation by lack of withdrawal upon firmly pinching a toe.
13. Lay mouse in supine position and pin down arms and legs with 22½ G needles.
14. Load 10 mL PBS into a 10 mL syringe with a 22½ G needle.
15. Dampen the abdomen and chest area with 70% ethanol spray.
16. With forceps, pull up the skin of the lower abdomen and using scissors, make a transverse incision to create a small opening into the abdominal cavity, and then make a midline incision in a cranial direction up to the level of the diaphragm.
17. Extend the midline incision cranially up to the neck, followed by lateral incisions (both directions) at the level of the clavicles and also at the level of the diaphragm after carefully separating it from the body wall. Expose the ribcage by setting aside the body wall flaps created by these incisions. Using scissors carefully cut along the sternum while angling upwards to avoid damaging the lungs and heart, followed by cutting of the ribcage along both sides of the chest to fully expose the lungs and heart.
18. Optional: For blood collection, use a 1 mL insulin syringe with a 26½ G needle to withdraw 0.4–1 mL of blood from the left ventricle (which should still be contracting).
19. Move the intestines gently to the right side (of the mouse) to expose the abdominal aorta. Transect the aorta to exsanguinate and absorb the blood with paper towels (there will be less blood after blood collection).
20. To remove blood from the lung vasculature, use forceps to hold on to the heart apex, and insert the 22½ G needle of the 10 mL syringe loaded with PBS (**step 14**) into the right ventricle of the heart, and inject the PBS until the lungs are maximally blanched.
21. If the heart is not contracting, it can be gently massaged using the forceps to assist perfusion of the lungs with the PBS.
22. Cut off the apex of the heart to drain the blood and prevent any blood from returning to the lung.
23. Optional: Lung lavage can be performed and BALF collected before removing the lungs by following the next six steps. If there is no need to collect either one, directly proceed to **step 30**.

24. To collect BALF, expose the trachea in the neck area and separate it from surrounding tissues using a surgical probe.
25. Insert the probe dorsal of the trachea to further separate it from the rest of the neck muscles, and lift it up to thread suture underneath the trachea.
26. After stabilizing the trachea with the suture, puncture it with a 22½ G needle directed caudally at a 45° angle (be careful not to puncture the dorsal wall of the trachea).
27. Insert an 18 G blunt-end needle or catheter through the opening made by the 22½ G needle in the trachea, and tie the surgical suture on the needle tip/trachea to hold it in place.
28. Fill a 1 mL syringe with 1 mL PBS, and attach it to the needle inserted in the trachea.
29. Lavage the lungs five times with 1 mL of PBS each time, and collect the BALF in a 15 mL tube. Promptly place on ice for further processing.
30. Promptly remove the lungs and place them on ice for further processing (*see Note 5*). The procedures after exsanguination should be undertaken as rapidly as possible to maximize recovery of intact mRNA and viable cells for further analysis.

3.2 Direct Endotracheal Injection of Bleomycin in Mice

1. The mice are anesthetized with a combination of ketamine and xylazine solution as described in Subheading 3.1 (step 2).
2. Shave the neck area and sterilize using betadine and alcohol swabs. Lay the mouse on the surgery board, and immobilize the tail and arms of the mouse with adhesive tape.
3. Using sterile forceps, pull up the skin in the sterilized neck area, and carefully make a 1 cm midline incision with sterile scissors taking care not to cut into underlying tissues. Expose the trachea through the incision using a sterile surgical probe.
4. Load a 1 mL syringe fitted with a 26 G needle with the appropriate amount of pre-diluted bleomycin or an equal volume of sterile PBS for controls. While directed caudally and at an oblique angle, carefully insert the needle into the visualized trachea; taking care not to go through to the dorsal wall of the trachea, rapidly inject the bleomycin solution during a single inspiration.
5. After withdrawing the needle, close the incision with an auto suture clip.
6. Place the animal on warmer pads to stabilize body temperature and allow recovery. Monitor until fully awake, commonly within an hour after induction of anesthesia.
7. Euthanize the mice for desired analysis at the time points of interest following the steps described in Subheading 3.1.

4 Notes

1. The C57BL/6 strain is commonly used because of its good responsiveness to bleomycin. Moreover abundant transgenic strains have this background. The doses in these protocols are based on usage of this strain. Older mice (>12–18 months) can be used in studies of age-dependent fibrosis, which would correspond to 60–70-year-old humans [63, 64]. The effects of bleomycin on gender are not clear and should be carefully considered. In general, the use of females may be advantageous for bleomycin studies because they do not cannibalize their sick littermates, and there is less fighting among the litter. However other considerations may trump this advantage.
2. Bleomycin is known to cause acute lung injury and subsequent fibrosis. Avoid inhalation of both the lyophilized and solubilized forms of bleomycin.
3. Because bleomycin is highly hygroscopic, it is recommended that the entire vial of lyophilized bleomycin be reconstituted at once to prevent the potential error of measurement.
4. Concentrations of anesthetic agents: dose ranges: ketamine (anesthetic) 80–120 mg/kg, xylazine 5–15 mg/kg. Mice and rats with different ages, genders, or genetic backgrounds have different responses to anesthetic drugs. The anesthetic dosages need to be optimized accordingly. The recommended doses of ketamine and xylazine used in C57BL/6 mice are 87 and 13 mg/kg, respectively.
5. Lungs can be inflated with fixative (e.g., 10% buffered formaldehyde) through the trachea before removing the lungs for further processing and use in histopathological examination, immunostaining, etc.

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