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REVIEW ARTICLE

Experimental mouse model of Bleomycin-induced Pulmonary Fibrosis

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ABSTRACT

Pulmonary fibrosis is a pathologic chronic lung disorder characterized by the deposition of extracellular matrix and damage to lung architecture. Models of pulmonary fibrosis were developed in several animal species. However, murine models are more common due to their low costs, availability and well characterized immune systems. Indeed, these murine models play an important role in understanding the biology of the diseases, deciphering the cellular and molecular mechanisms mediating pulmonary pathobiology and to explore novel therapeutic approaches. Although the majority of these models mimic certain characteristics of human idiopathic pulmonary fibrosis, most of them do not mimic other features especially those related to the progressive and irreversible nature of this disease. The present protocol describes the induction of a pulmonary fibrosis model in mice with bleomycin, which is a risk factor for human lung fibrosis. Specifically, we described the methodology of intratracheal instillation of bleomycin as well as the assessment methods of pulmonary fibrosis either histopathologically based on Ashcroft grading score or by quantitatively based on biochemical biomarkers. Moreover, we proposed a detailed readout that can determine the degree of lung injury in bronchoalveolar lavage fluids. We described different cellular and molecular markers such as by total protein concentration, total cell count, immune cell differential count, and the expression of pro-inflammatory mediators. We present this model as a reference for studies on the pulmonary fibrosis.

Keywords : Bleomycin, Bronchoalveolar lavage, Cytokines, Histopathology, Biochemical mediators, Intratracheal, Mice, Murine models, Pulmonary fibrosis

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INTRODUCTION

Pulmonary fibrosis is a chronic lung disorder, which is pathologically characterized with an increase in accumulation of extracellular matrix (ECM) and permanent damage of lung architecture due to injury-induced scar formation. This leads to organ failure, disturbance in gas exchange, and death from respiratory malfunction (King et al., 2011). Several types of pulmonary fibrosis have been described by pathological analysis including; diffuse fibrosing alveolitis, diffuse interstitial fibrosis, and idiopathic pulmonary fibrosis (IPF). Currently, IPF is considered the most common and fatal form of chronic pulmonary fibrosis (Sauleda et al., 2018). This disease is characterized by progressive dyspnea, bilateral interstitial infiltrates, and restrictive physiology on pulmonary function testing (Degryse and Lawson 2011).

Pulmonary fibrosis can be developed after viral infections (Kelly et al., 2002) and after exposure to radiotherapy (Denham and Hauer-Jensen 2002), chemotherapeutic drugs (Chen and

Stubbe 2005), and aerosolized environmental toxins (Fubini and Hubbard 2003). Different types of pulmonary fibrosis were successfully modeled and studied in rodents using drugs (e.g. bleomycin) (Moeller et al., 2008), particulate matter (e.g. asbestos and silica), fluorescein isothiocyanate (FITC), irradiation, delivery of transgenes using a viral vector or transgenic mice using fibrogenic cytokines (Tashiro et al., 2017; Degryse and Lawson 2011). Indeed, these murine models play an important role in understanding the biology of the diseases, deciphering the cellular and molecular mechanisms mediating pulmonary pathobiology and to explore novel therapeutic approaches. Although the majority of these models mimic certain characteristics of human idiopathic pulmonary fibrosis, most of them do not mimic other features especially those related to the progressive and irreversible nature of this disease (Tashiro et al., 2017). The perfect animal model would replicate the findings noted in IPF including the histological features of usual interstitial pneumonia (UIP), be progressive and irreversible, applicable across mouse strains, inexpensive, and be reproduced in a short time frame (Moore and Hogaboam 2008).

The pathogenesis of IPF is not yet well understood. Several cycles of lung injury have been believed to destroy alveolar epithelial cells, leading in turn to the migration, development, and activation of mesenchymal cells as well as the excessive accumulation of fibroblasts and myofibroblasts. This leads to excessive deposition of collagen in the lung interstitium and alveolar space, which represents aberrant wound repair (Ruscitti et al., 2017). The present protocol describes the induction of a pulmonary fibrosis model in mice with bleomycin, which is a risk factor for human lung fibrosis.

BLEOMYCIN ANIMAL MODEL

Bleomycin is a chemotherapeutic agent that has been known to induce pulmonary fibrosis as an infrequent side effect in humans who receive it for treating of various cancers (Degryse and Lawson 2011). The histological hallmarks, such as intra-alveolar fibrosis, deposition of collagen and destruction of the alveolar space, has been found in bleomycin-treated animals similar to IPF patients (Usuki and Fukuda 1995). This finding has led to the assumption that bleomycin mimics the typical features of human pulmonary fibrosis disease. Furthermore, the bleomycin model is easy to use, widely accessible and reproducible, thus fulfilling important criteria expected from a good applied animal model of lung fibrosis (Moeller et al., 2008).

Experimentally, bleomycin can be given directly into the airway by intratracheal, intranasal or inhalation routes or systemically via subcutaneous, intraperitoneal, or intravenous injection. Bleomycin intratracheal administration model is the most common because of its ease of delivery and a short time to induce fibrosis. This drug can be administered via surgical tracheal cut-down or through endotracheal intubation (Moore and Hogaboam 2008). The response to bleomycin is strain-dependent; CBA and C57Bl/6 mice strains are more susceptible to the development of pulmonary fibrosis than BALB/c mice that are comparatively fibrosis-resistant. This discrepancy is mainly due to variation in the development of the inactivated bleomycin hydrolase between strains. The lungs that have low levels of this enzyme are more likely to induce tissue injury and fibrosis by bleomycin administration (Moeller et al., 2008).

Regardless the route of administration, bleomycin contributes to a direct cell injury through the initiation of breakdowns in DNA strand, free radicals' production, and oxidative stress activation. The bleomycin model consists of two different phases: the inflammatory phase that occurs within 2 weeks of the injury and then decreases during the fibrotic phase (Ruscitti et al., 2017). Cell necrosis and apoptosis are followed with intense inflammation and fibrotic reactions within a short period of time (Degryse and Lawson 2011). The initial elevation of the pro-inflammatory cytokines (TNFα, IL-1, and IL-6) occurs during the first week and the expression of the profibrotic markers (TGFβ1, fibronectin, and collagen peaks around day 14 post bleomycin instillation. By the second- and third-week post bleomycin adminstration, patchy of isolated collagen deposits developed

resembling fibrosis with prominent deposition of ECM components including fibronectin and collagen I (Moore et al., 2013).

It is now become evident that interventions occurring during the inflammatory phase are often protective and not expected to translate into a clinical benefit. Therefore, to determine the efficacy of an antifibrotic drug in bleomycin model, it is recommended to deliver the therapy during the fibrotic phase (at least 7 days post-bleomycin after the inflammatory phase) (Carrington et al., 2018). Many other bleomycin regimes have been used to make a better model that mimics the progressive nature of IPF with repeated lower doses of bleomycin delivered both locally to the lungs (Peng et al., 2013) or systemically (Moore and Hogaboam 2008).

The bleomycin animal model is considered a valuable research tool for the elucidation of cells, mediators, and signaling pathways that contribute to pathogenesis of pulmonary fibrotic disorders. Additionally, it provides an important preclinical model for the testing the potential effect of various antifibrotic drugs (Moore and Hogaboam 2008; Moore et al., 2013). A significant number of compounds in this model have been demonstrated to prevent fibrotic progression and are proposed to qualify for clinical use (Moeller et al., 2008).

BLEOMYCIN-INDUCED LUNG FIBROSIS

Equipment and materials

- Fiber Optic Illuminator
- Fiber Optic Stylets
- Endotracheal Tubes
- Lung Inflation Bulb
- Intubation Stand
- Rubber Wire
- Forceps
- Metric weight scale
- Catheter
- Ketamine
- Xylazine
- Bleomycin (APP Pharmaceuticals, Schaumburg, IL, USA, #63323013720)

Protocol Steps

- Sedate adult mice between 8 and 10 weeks of age with anesthesia containing ketamine (10 mg/ml) and xylazine (1.25 mg/ml).
- Suspend each mouse on the intubation stand using the rubber wire from its incisors as shown in (Figure 1A).
- With forceps, gently retract the tongue and insert the end of the endotracheal tube with fiber optic stylet into trachea guided with fiber optic illuminator.
- The catheter should be noticeable while moving down the trachea approximately 5 mm and passing the vocal cords but still far above the carina. The catheter's movement will not be visible if it goes down into the esophagus.
- Once the catheter is seen in the trachea in the neck, administer intratracheally a single dose (1U/kg) of bleomycin in PBS or PBS alone as a control through the catheter with a loading pipette (Koo et al., 2018).
- Using the inflation bulb through the catheter, rapidly inflate the lungs with air so that the liquid is evenly distributed in the lungs (Figure 1B).
- Monitor the mice daily and record their weights twice weekly. Mice with >20% weight loss or significant respiratory distress will be humanely euthanized and treated like a death.

LUNG EXCISION AND FIXATION FOR HISTOLOGICAL EXAMINATION

Equipment and materials

- Dissecting board with cork or rubber surface
- Spray bottle with 70% ethanol
- Pins
- Forceps
- Scissors
- 10 ml syringe with 23 g needle
- 20G-3" stainless straight needle with 2.25mm ball tip (Braintree Scientific, #N-PK 003)
- Silk suture thread (Braintree Scientific, #SUT-S 108)
- 1X PBS
- 10% phosphate-buffered formalin
- Xylene
- Paraffin
- Razor blade
- Tissue cassettes
- Pencil or solvent-resistant marker

Protocol Steps

- Euthanize mice interperitoneally with an anesthetic overdose of ketamine/xylazine cocktail (500-700 µl/ mouse).
- Fix the mouse on the dissection board and dampen the fur with 70% ethanol.
- Using scissors, dissect the mouse by making a small incision just below the rib cage and cut through the skin and muscles upwards until exposing the thoracic cage and the neck.
- Perform a tracheostomy by making a small semi-excision in the trachea using a small scissors.
- Insert a 20G-3" stainless straight needle with 2.25mm ball tip into the trachea and firmly tie together the needle and the trachea with a silk suture thread.
- Slowly perfuse the lungs with 5 ml cold PBS via the right ventricle using a 10 ml syringe until lungs cleared of blood and changed to white color. Cut down the abdominal aorta to allow the blood to leave.
- Fix the whole lung lobes via intratracheal inflation with 10% phosphate-buffered formalin at a pressure of 20 cm water for 10 minutes (Figure 2), followed by immersion in fresh fixative overnight at room temperature.
- Wash out the lungs with 1X PBS for 15 minutes with agitation 3 times.
- Dissect the lung lobes in a petri dish using a sharp razor blade then transfer the lobes to labeled cassettes and keep in 70% ethanol at 4°C until processing.
- Dehydrate the samples in ethanol, clear in xylene, and then embed in paraffin.

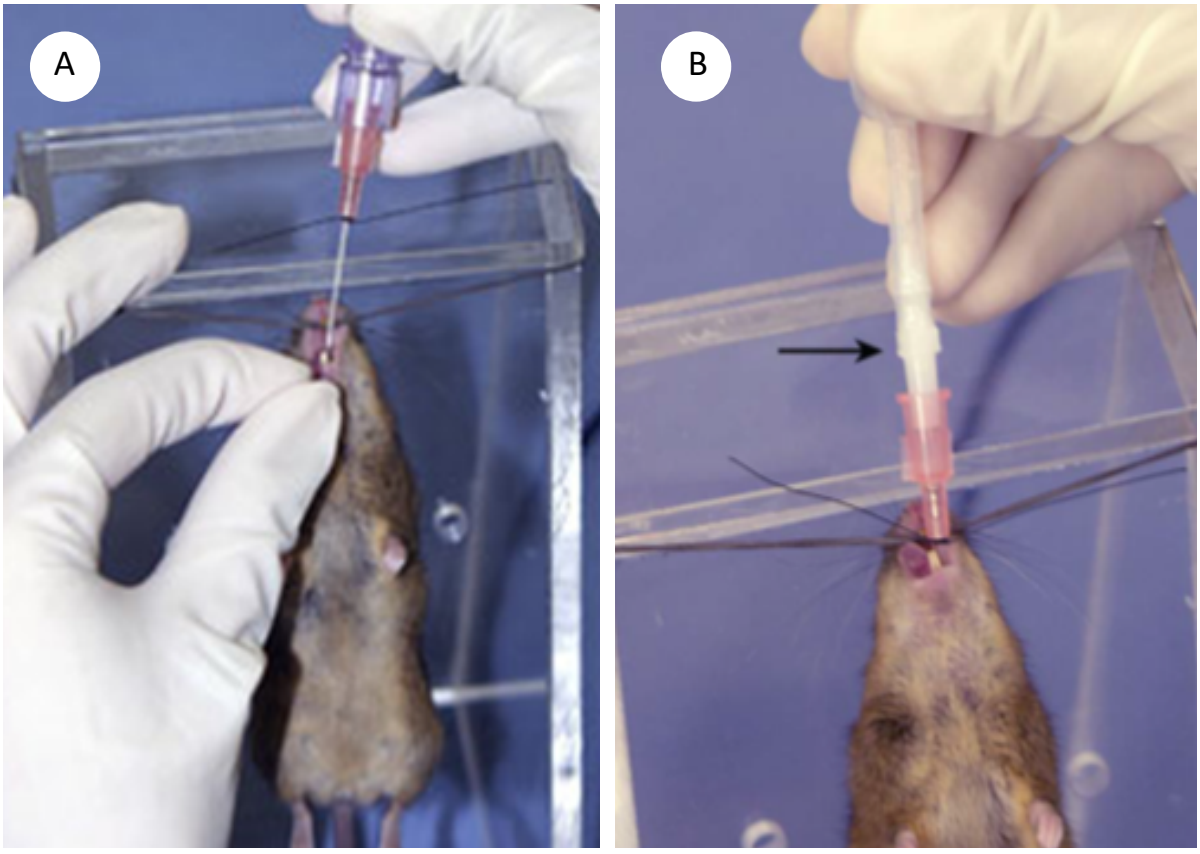

Figure 1. *Oral intubation of mice using a BioLite intubation kit*. **A)** Retraction of the tongue and insertion of the intubation system. **B)** Verification of bleomycin instillation by doing rapid inflation of air into the lungs using the inflation tube (Rivera *et al.* 2005).

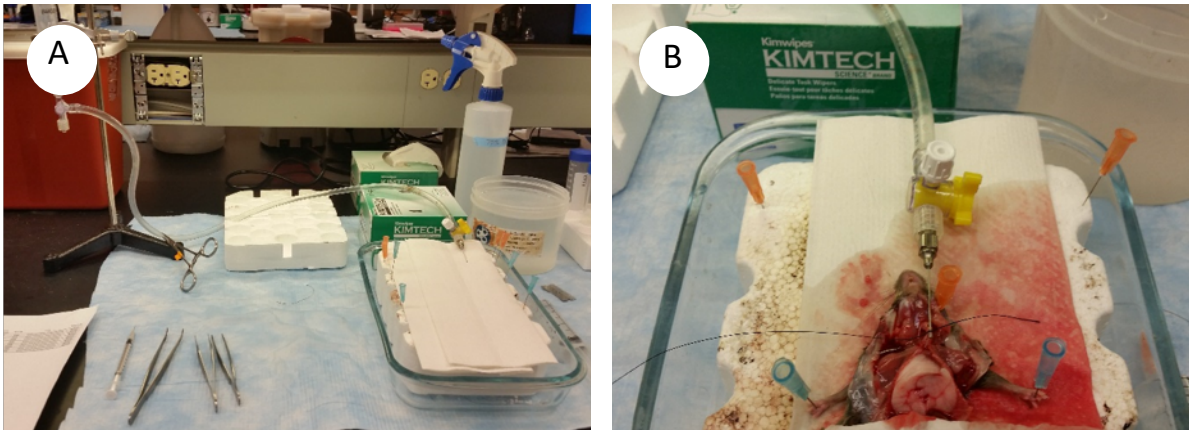

Figure 2. *Fixation of mouse lung.* **A)** Formalin fixation apparatus. **B)** Lung-filled with formalin during fixation.

ASSESSMENT OF LUNG FIBROSIS

There are several common methods used to assess fibrosis, including semi-quantitative histopathological analysis, based on the Ashcroft scoring system (Ashcroft *et al.,*1988), and quantification of collagen content by hydroxyproline assay (Moeller *et al.,* 2008).

Histopathological analysis

- Stain 5 µm sections either by H&E (to assess lung injury and inflammation) (Figure 3 A-D) or by Masson's trichrome (to assess lung fibrosis) (Figure 3 E, F).
- Score individually 20 non-overlapping of 10× fields per sample for the severity of interstitial fibrosis using the Ashcroft scoring scale.
- Assess separately each successive field for severity of interstitial fibrosis and give a score between 0 and 8 and the average score of all fields indicates the Ashcroft score of a single sample. The scoring scale is as follows: 0 = no abnormalities, 1 = slight thickening of alveolar membranes, 2 = small areas of fibrosis (<10%), 3 = 10-20% fibrotic area, 4 = 20-40% fibrotic area, 5 = 40-60% fibrosis, 6 = 60-80% fibrosis, 7 = >80% fibrosis, and 8 = complete fibrosis (Ashcroft, Simpson, and Timbrell 1988).

Hydroxyproline assay

The hydroxyproline assay method is performed as described previously (Kliment *et al.,* 2011; Kach *et al.,* 2013) but with some modifications.

Materials and reagents

- 14ml round-bottom Falcon tube (Fisher Scientific, #352059)
- 1.5 ml microcentrifuge tubes
- Cap locks (Fisher Scientific, #NC9679153)
- 96-well plate
- 12 N hydrochloric acid (HCl; Fisher Scientific, #A144-500)
- Citric acid (Sigma-Aldrich, #251275)
- Glacial acetic acid (Fisher Scientific, Fair Lawn, NJ, USA, #BP1185)
- Sodium acetate anhydrous (Fisher Scientific, #BP333)
- Chloramine T trihydrate (Sigma-Aldrich, #402869)
- n-propanol (Fisher Scientific, #A414)
- 4-(Dimethylamino) benzaldehyde (Sigma-Aldrich, #156477)
- Perchloric acid (Sigma-Aldrich, #31142)
- 4-Hydroxy-L-proline (Hyp) (Sigma-Aldrich, #41875)
- NaOH
- dH2O

Reagents preparation

- **Citrate-acetate buffer (500 ml):** Citrate-acetate buffer solution is formed of 5% citric acid, 1.2% glacial acetic acid, 7.24% sodium acetate anhydrous, 3.4% NaOH, and dH_2O (pH=6.0) to reach the total volume 500 ml.
- **Chloramine T :** Chloramine T is formed of 0.282 g Chloramine T trihydrate, 2 ml n-propanol, 2 ml dH_2O, and citrate-acetate buffer is added to 20 ml line.

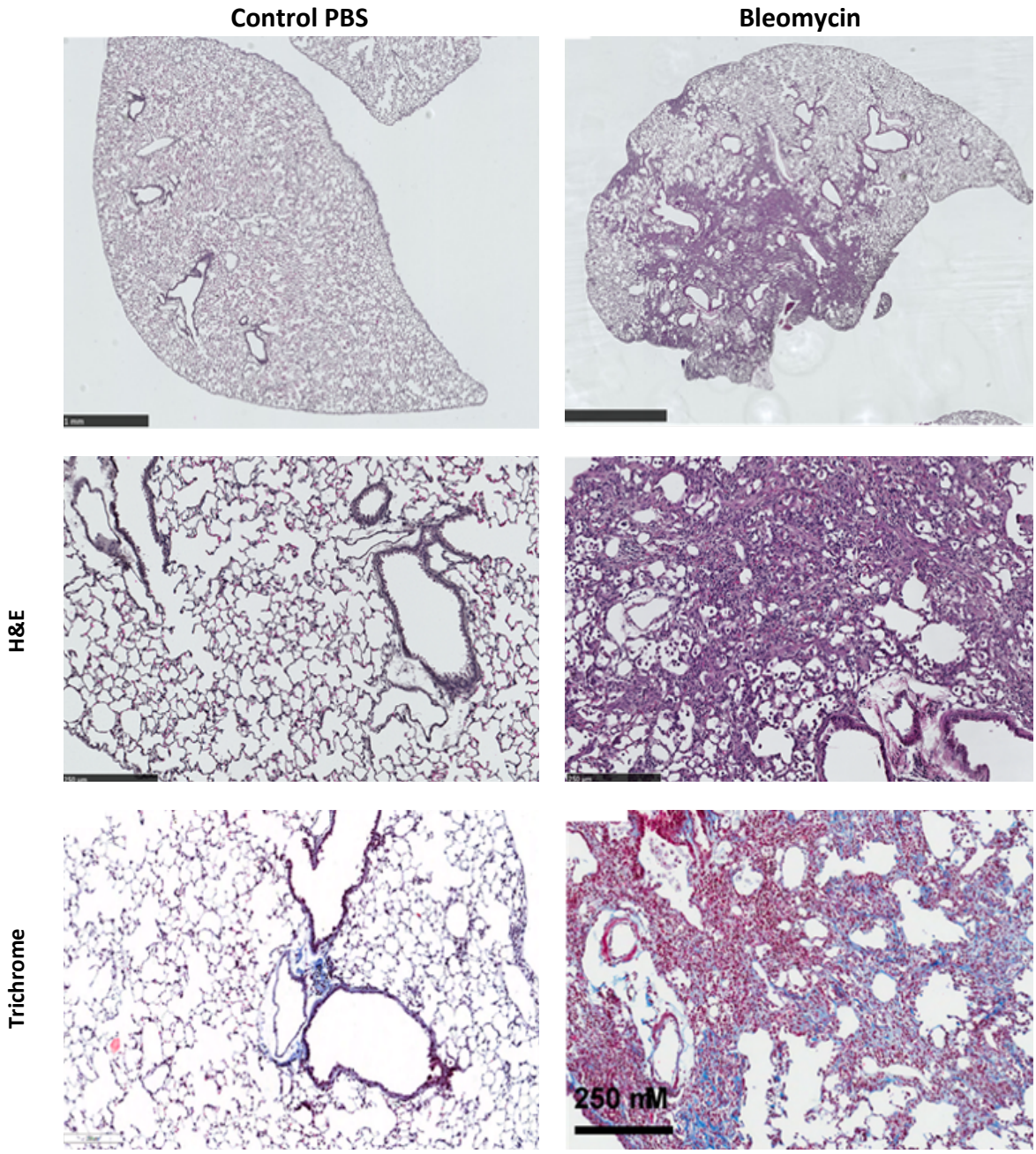

Figure 3. Representative images showing H&E (A-D) and Masson's Trichrome (E, F) staining of control and fibrotic lung post-bleomycin; 1.2 × (scale bar = 2.5 mm) and 10 × (scale bar =250 µm) magnified images are shown.

- **Ehrlich's solution (This must be made fresh):** Ehrlich solution is made of 4.5 g 4-(Dimethylamino) benzaldehyde, 2 ml n-Propanol, and 7.8 ml 70% perchloric acid.

Tissue preparation/hydrolysis

- Homogenize the right lung of samples in 1 ml of cold PBS in 14ml round-bottom Falcon tube at max speed for 20-30 seconds.
- Transfer 500 µl of homogenate to a new microcentrifuge tube and add 500 µl of 12 N hydrochloric acid in a 1:1 ratio.
- Seal each tube with cap locks and hydrolyze overnight at 110°C in a vacuum oven.
- Leave the samples to cool down to room temperature, spin down at max speed for 30 seconds, vortex and then spin down again.

Sample and standard processing

- Prepare the hydroxyproline standards is in PBS (each in duplicate or triplicate) from 1 mg/ml stock solution of 4-Hydroxy-L-proline (Hyp) with serial dilutions as indicated below.
- Add 10 µl of each standard or hydrolyzed sample in 96-well plate in triplicate overnight under a fume hood to evaporate and remove excess HCl.
- Reconstitute the samples with 100 µl of freshly made Chloramine T and leave at room temperature for 20 minutes.
- Add 100 µl of freshly made Ehrlich's reagent to each well, and incubate the plate at 65°C for 15 minutes.
- This method gives an orange-red color then measure the absorbance measured at 550 nm and compare to a hydroxyproline standard curve generated from pure hydroxyproline.

Calculations

- Calculate the amount of hydroxyproline in each well using the standard curve, then average the concentration of each sample.
- Determine the hydroxyproline concentration in the entire 1 ml hydrolysate by multiplying the average number of each sample by 100 (each well contains 10 µl, but the entire sample is 1000 µl. 1000µl/10µl=100).
  Hydroxyproline (mg) of hydrolysate = (Hyp conc. per well X 1000 ml hydrolysate) / 10 ml added per well
- Determine the total hydroxyproline concentration in 1 ml homogenate of the right lobe by multiply results of step 2 by 2 (the hydroxyproline hydrolysate was made from 500 µl of the total 1000 µl homogenate. 1000µl/500µl=2).
  Hydroxyproline (mg) of homogenate = Hyp of hydrolysate X 2

BRONCHOALVEOLAR LAVAGE FLUID (BALF)

Bronchoalveolar lavage fluid (BALF) is often analyzed for changes in total protein concentrations, total cell count, differential count of inflammatory immune cells either by using cytospin slide preparations with Wright-Giemsa stain or by using flow cytometry, and measurement of the expression levels of pro-inflammatory mediators. Additional parameters such as weight, lung index (wet lung weight in milligrams versus body weight in grams), wet: dry ratio and survival time are not always, but frequently assessed. Those method are used as an assessment of the degree of lung injury (Moeller *et al.,* 2008).

Equipment and materials

- Dissecting board with cork or rubber surface
- Spray bottle with 70% ethanol
- Pins
- Forceps
- Scissors
- 20G-3" stainless straight needle with 2.25mm ball tip (Braintree Scientific, #N-PK 003)
- Silk suture thread (Braintree Scientific, #SUT-S 108),
- Insulin syringes
- 1X PBS
- 10 mM EDTA
- Ammonium-Chloride-Potassium (ACK) lysis buffer (Thermo Scientific, Rockford, IL, USA, #A1049201)
- Protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA, #P8340)
- Trypan blue stain (BioRad, Hercules, CA, USA, #1450022)
- Microcentrifuge tubes
- Hemocytometer slide
- permanent marker for labeling
- Centrifuge
- Microscope or automated cell counter

Protocol Steps

- Euthanized mice with an overdose of ketamine and xylazine and exsanguinate by cutting the abdominal aorta.
- Dissect the trachea and cannulate as previous, then lavage the lung with 1ml of 1X PBS + 10 mM EDTA three times using an insulin syringe.
- Record the total volume of BALF, and transfer to a labeled microcentrifuge tube, then centrifuge at 500xg for 5 minutes at 4°C. If red blood cells are present, add 500 µl of ACK lysis buffer and re-pellet the cells.
- Transfer the supernatant to a new tube and add the protease inhibitor cocktail to a final

concentration of 1X, mix well and store at -80°C until processing.

- Re-suspend the cell pellet in 100 µl PBS and count the total cell number on a hemocytometer slide using trypan blue exclusion under a microscope or with automated cell counter.

Differential count of immune cells using flow cytometer

- Protocol Steps
- Anesthetize the mice and dissect as above.
- Expose the trachea, cannulate and tighten with suture.
- Fill the lungs with cold PBS + 1 mM EDTA three times.
- Centrifuge the samples at 450xg for 5 minutes at 4°C.
- Lyse red blood cells using 500 µl of ACK lysis buffer, then pellet.
- Resuspend the cells in 100 µl of staining buffer (1x PBS + 1% BSA + 0.1% sodium azide) containing Fc block (1:100) and incubate on ice for 5-10 minutes.
- Take 10 µl from each sample to determine total cell count.
- Combine 15 µl from each sample in a separate tube and aliquot evenly to tubes for single fluorophore and unstained controls.
- Prepare antibody master mix in staining buffer of fluorophore-conjugated antibody (final conc. of each Ab was 1:200) including; Siglec-F, CD11c, CD11b, F4/80, Ly-6G, CD3 and MHCII (Table 1), then re-suspend the cell pellets in 200 µl of antibody mix.
- Incubate the samples on ice for 30-45 minutes and protect from light.
- Wash the cells two times with 1.5 ml of staining buffer, then pellet at 500xg for 5 minutes at 4°C between each wash.
- Measure the cell subsets using a BD LSR II flow cytometer and analyze the data using FlowJo software as follows:
    - T-cells ($CD3^+$)
    - B-cells (SSC^{low} $MHCII^+$)
    - Eosinophils (Siglec-F^+ $CD11c^{low}$)
    - Alveolar Macrophages (Siglec-F^+ $CD11c^+$ $F4/80^+$ $CD11b^{Int/low}$)
    - Monocyte-derived Macrophages (Siglec-F^+ $CD11c^+$ $F4/80^+$ $CD11b^{high}$)
    - Interstitial Macrophages (Siglec-F^- $CD11c^-$ $CD11b^+$ $F4/80^+$)
    - Monocytes (SSC^{low} $CD11b^+$)
    - Neutrophils (Siglec-F^- $CD11c^-$ $CD11b^+$ Ly-$6G^+$)
    - Dendritic cells ($CD11c^{high}$ $MHCII^{high}$)

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EACR is an NGO society that was declared by the Ministry of Social Solidarity (Egypt) No. 1938 in 19/11/2014 based on the initiative of Prof. Mohamed Labib Salem, the current Chairman of EACR. EACR aims primarily to assist researchers, in particular young researchers in the field of cancer research through workshops, seminars and conferences. Its first international annual conference entitled "Anti-Cancer Drug Discovery" was successfully organized in April 2019 (http://acdd.tanta.edu.eg). Additionally, EACR aims to raise the awareness of the society about the importance of scientific research in the field of cancer research in prediction, early diagnosis and treatment of cancer. EACR is also keen to outreach the scientific community with periodicals and news on cancer research including peer-reviewed scientific journals for the publication of cutting-edge research. The official scientific journal of EACR is "International Journal of Cancer and biomedical Research (IJCBR: https://jcbr.journals.ekb.eg) was successfully issued in 2017 and has been sponsored by the Egyptian Knowledge Bank (EKB: www.ekb.eg).

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