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Exploring the Impact of Molecular Hydrogen Therapy on Oxidative Stress in Bleomycin-Induced Idiopathic Pulmonary Fibrosis: A Research Protocol

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Abstract

Introduction: Idiopathic Pulmonary Fibrosis (IPF) is an intensifying respiratory disorder triggered by bleomycin in cancer patients, characterized by its lack of a definitive remedy. Bleomycin induces mitochondrial leakage and elevates Reactive Oxygen Species (ROS) in the pulmonary cavity, causing a reduction in glutathione (GSH), a vital lung antioxidant. This reduction in GSH levels, coupled with ROS's inactivation of the tumor suppressor gene p53, increases the chance of developing fibrosis. Although no antioxidant treatments that currently target these effects, Molecular Hydrogen Therapy (MHT) has proven effective in mitigating ROS in diseases like Chronic Obstructive Pulmonary Disease (COPD). This study aims to explore the impact of bleomycin-induced IPF on ROS and p53, investigating MHT as a potential treatment to alleviate oxidative stress in fibrosis.

Methods: The experimental design includes four groups of mice, two receiving bleomycin injections and two receiving a control buffer solution. Within each subgroup, one group will undergo MHT, while the other will receive sterile air. Bronchoalveolar lavage will be used to measure GSH levels before and after MHT, and RNA sequencing will monitor p53 activity in all treatment and control groups.

Results: Anticipated outcomes include elevated GSH levels in the MHT-treated bleomycin group, indicating an antioxidative effect. Consequently, improved p53 activity is expected compared to controls.

Discussion: Anticipated results, based on existing literature, suggest that MHT enhances antioxidant defenses and modulates p53 activity, thereby reducing oxidative damage and fibrotic remodeling. These findings highlight the potential of MHT as a therapeutic intervention for IPF, with broader implications for managing other oxidative stress-mediated lung diseases. However, further research, including preclinical studies and clinical trials, is needed to validate these anticipated findings and ensure the long-term safety and efficacy of MHT in clinical practice.

Conclusion: The study aims to replicate findings in human populations and proposes a paradigm shift in treating such diseases by addressing the root cause, ROS.

Keywords: idiopathic pulmonary fibrosis; reactive oxygen species; RNA sequencing; molecular hydrogen therapy; p53

Introduction

Idiopathic Pulmonary Fibrosis (IPF) is a chronic, progressive lung disease characterized by the gradual scarring and thickening of lung tissue, leading to a decline in lung function and respiratory failure [1]. IPF is known to be an irreversible and fatal disease [2]. In cancer patients, particularly those undergoing chemotherapy, the use of bleomycin, a cytotoxic antibiotic, has been associated with the development of Drug-Induced Pulmonary Fibrosis (DIPF), a condition reminiscent of IPF [3].

Despite being a rare complication, DIPF poses significant challenges due to its high morbidity and mortality rates, as well as the absence of effective treatment strategies [4]. DIPF, though a rare complication, poses significant challenges due to its high morbidity and mortality rates. The disease has a reported 5-year mortality rate of approximately 50% to 70%, highlighting its severe prognosis [5-7]. Furthermore, the median survival time for patients diagnosed with DIPF is often cited as 2 to 5 years from the time of diagnosis, underscoring the urgency for more effective treatment strategies [7, 8].

Bleomycin-induced pulmonary fibrosis (B-IPF) shares similarities with IPF regarding histopathological features, including interstitial fibrosis, inflammation, and honeycombing patterns in imaging studies [9].

The pathogenesis of DIPF involves bleomycin-induced oxidative stress, DNA damage, and inflammation, leading to the activation of fibroblasts and aberrant wound-healing

responses in the lungs [10] as shown in Figure 1 below. Bleomycin exerts its cytotoxic effects by generating reactive oxygen species (ROS), which induces DNA strand breaks within lung tissue [11] as shown in Figure 1. ROS, including superoxide radicals and hydrogen peroxide, inflict oxidative damage to cellular components such as DNA, proteins, and lipids [12]. This oxidative stress triggers a cascade of events that contribute to the pathogenesis of pulmonary fibrosis [10] as shown in Figure 1.

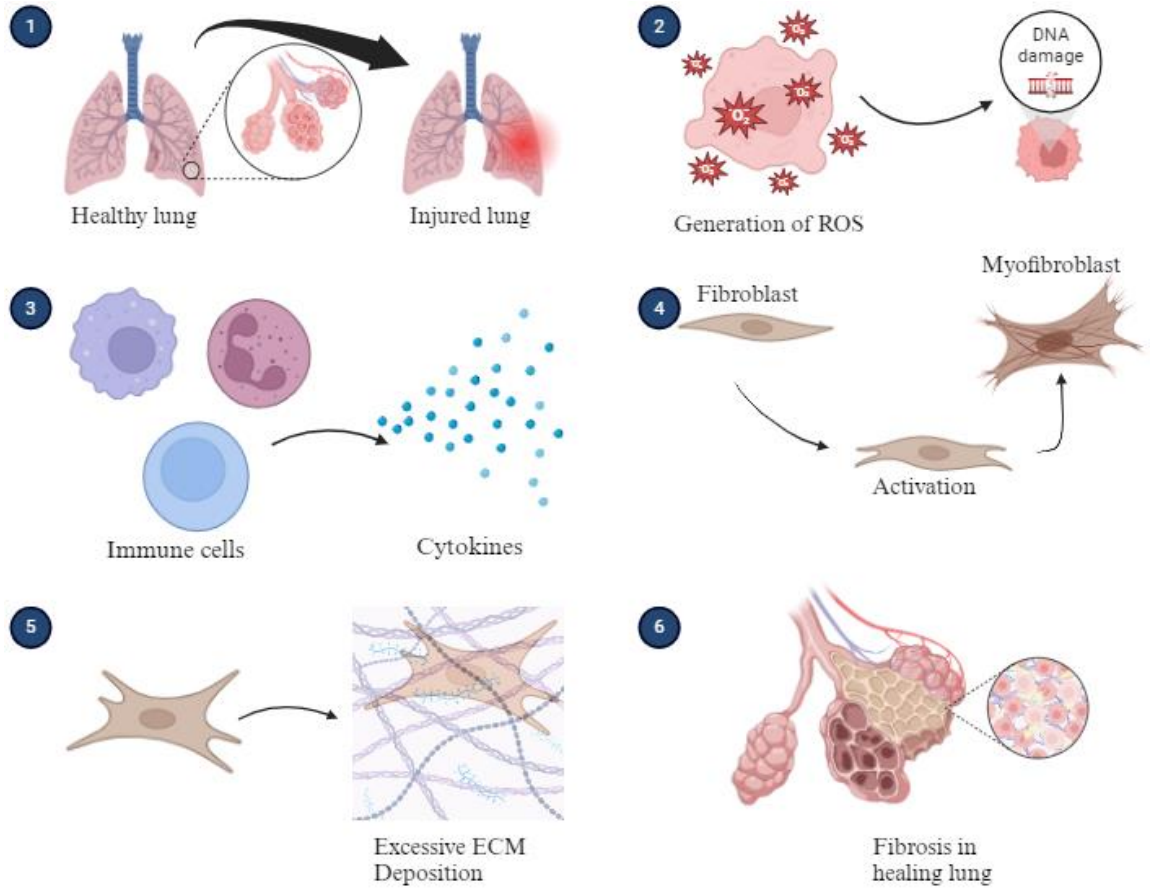

Figure 1. An illustration of the cascade of events that contribute to the pathogenesis of pulmonary fibrosis. 1) Initial lung injury, 2) Generation of ROS resulting in DNA damage, 3) Immune cells releasing cytokines, 4) Fibroblast activation and transformation into myofibroblasts 5) Excessing extracellular matrix (ECM) deposition by myofibroblasts, 6) Fibrosis. Figure made with BioRender.

The tumor suppressor gene, p53, is central to this cascade, which plays a crucial role in orchestrating cellular responses to stress and DNA damage [13]. In response to bleomycin-induced ROS, p53 activates various cellular processes including DNA repair, cell cycle arrest, and apoptosis [14]. While activation of p53 initially serves to limit DNA damage and promote cellular repair, sustained activation can lead to aberrant signaling and exacerbate tissue injury and inflammation, ultimately driving fibrotic remodelling [10, 14].

The antioxidant glutathione (GSH) is another crucial player in the response to bleomycin-induced oxidative stress [15]. GSH is a potent scavenger of ROS, neutralizing free radicals and protecting cells from oxidative damage [15]. Reduced glutathione (GSH) is a naturally available and abundant antioxidant which reduces reactive oxygen species (ROS) as it has thiol groups that can donate electrons. GSH in the process becomes oxidized glutathione, which consists of 2 GSH molecules linked by a disulphide bond (GSSG). Therefore; measuring total Glutathione (GSH + GSSG), provides the overall glutathione content, reduced Glutathione (GSH) indicates the available antioxidant capacity, oxidized Glutathione (GSSG) indicates the level of oxidative stress and the GSH/GSSG Ratio is comprehensive indicator of the cellular redox balance [16]. However, in elevated ROS levels generated by bleomycin, GSH becomes oxidized and depleted. This depletion compromises the lung's antioxidant defenses, further exacerbating oxidative stress and contributing to the progression of pulmonary fibrosis [17].

The intricate relationship between bleomycin, p53, GSH levels, and ROS underscores the complex interplay

between oxidative stress, DNA damage, and cellular responses in the pathogenesis of pulmonary fibrosis. Understanding these mechanisms is crucial for developing targeted therapeutic interventions to mitigate oxidative stress and preserve lung function in patients exposed to bleomycin-induced pulmonary fibrosis. Strategies aimed at restoring GSH levels or enhancing antioxidant capacity may hold promise for attenuating oxidative damage and preventing the progression of pulmonary fibrosis in affected individuals.

Management of DIPF, specifically, revolves around early recognition and discontinuation of bleomycin therapy to prevent further lung injury [18]. However, once fibrosis has developed, treatment options are limited. Corticosteroids, the mainstay of therapy in other forms of drug-induced lung injury, have shown limited efficacy in DIPF and may even exacerbate fibrosis in some cases [19]. Researchers have evaluated other pharmacological interventions, such as pirfenidone and nintedanib, in IPF which have shown promise in preclinical models of bleomycin-induced fibrosis [20]. However, their efficacy and safety in the context of DIPF remain unclear, and further research is needed to elucidate their role in this setting.

Given the lack of effective treatments for DIPF, supportive measures to manage symptoms and improve quality of life are crucial. One promising approach is Molecular Hydrogen Therapy (MHT), which has shown effectiveness in reducing oxidative stress and inflammation in Chronic Obstructive Pulmonary Disease (COPD). MHT involves the administration of molecular hydrogen (H_2), a potent antioxidant that selectively neutralizes harmful ROS such as hydroxyl radicals and peroxynitrite, without affecting beneficial reactive species involved in normal cellular signalling [21]. This selectivity is crucial, as it allows for the mitigation of oxidative damage without disrupting physiological ROS-dependent processes.

In patients with COPD, MHT has demonstrated the ability to alleviate lung inflammation and improve pulmonary function. Studies have shown that inhalation of hydrogen gas can significantly decrease levels of inflammatory markers and oxidative stress in the lungs, leading to improved respiratory outcomes [22]. The mechanism behind MHT's efficacy lies in its antioxidant properties; by scavenging excessive ROS, MHT prevents the oxidative damage to cellular components such as DNA, proteins, and lipids, thereby reducing inflammation and promoting cellular health [21, 22].

The therapeutic potential of MHT extends beyond COPD and is being explored in various oxidative stress-related diseases. Its application in pulmonary fibrosis, particularly bleomycin-induced pulmonary fibrosis, is of great interest due to the shared pathological mechanisms involving oxidative stress and inflammation. MHT could potentially mitigate the oxidative damage induced by bleomycin, protect lung tissues from further injury, and improve overall lung function [17].

This study seeks to investigate the effects of bleomycin-induced IPF (B-IPF) on ROS and p53 while exploring the potential of MHT as a treatment option to mitigate oxidative stress in fibrosis in animal models. We hypothesize that treatment with MHT in the bleomycin-induced lung injury murine model will result in elevated levels of GSH compared to the control group, indicating an antioxidative effect. Consequently, we expect to observe improved p53 activity in the MHT-treated group compared to controls.

Methods

Experimental Groups and Controls

Four distinct groups of mice will be established: Group 1: PBS control group (n=10): Mice will be administered to an equivalent volume of phosphate-buffered saline (PBS). Group 2: PBS + MHT control group (n=10): Mice will be administered to an equivalent volume of PBS and Molecular Hydrogen Therapy. Group 3: Bleomycin group (n=10): Mice subjected to bleomycin injections. Group 4: Bleomycin + MHT group (n=10): Mice subjected to bleomycin injections and administered Molecular Hydrogen Therapy.

Group 1 is a negative control to assess baseline levels of oxidative stress (in the absence of bleomycin) due to normal levels of GSH and p53 activity. This helps consider any inherent variability caused in the measurements of this experiment that are unrelated to the treatments administered.

Group 2 receives the same amount of PBS and MHT. This helps evaluate the potential impacts of MHT on baseline levels of oxidative stress (in the absence of bleomycin). Another major role of this group is to establish whether the effects of MHT are specific to fibrosis or can be seen only in drug-induced fibrosis. This also helps observe any nonspecific effects of MHT, or side effects of MHT.

Group 3 consists of mice treated with Bleomycin to induce pulmonary fibrosis. These mice are not subject to any protective treatment. Group 4, on the other hand, is treated with both Bleomycin to induce pulmonary fibrosis, followed by MHT treatment as protective and therapeutic. The comparison between these two groups will help assess the efficacy of MHT in mitigating the effects of oxidative stress and p53 activity.

The function of the two control groups is to provide baseline comparison and increase the validity and reliability of the results obtained from this study [23]. The two groups offer substantially essential reference points for data interpretation, distinguishing environments that are treatment-specific from baseline environments and ensuring specificity and validity of the observed outcomes of MHT implementation related to oxidative stress and p53 activity.

Preparation of Solutions Used in the Study

Preparation of Sterile PBS

The required volume of phosphate-buffered saline (PBS) will be measured. Typically, each mouse will be given 100µl of PBS [24].

PBS will be filtered using a 0.22-micron filter unit attached to a sterile syringe or filtration system. This is to remove any particulate contaminants and microorganisms, ensuring the PBS is sterile.

Sterility Confirmation

After filtration, a small sample of the PBS will be taken and plated on an agar plate. This plate will then be Incubated at 37°C for 24-48 hours to check for microbial growth. No microbial growth indicates sterility. [25]. This filtration process will guarantee that the PBS is free from any contaminants or microorganisms, thereby ensuring the integrity of the final bleomycin sulfate solution. This approach to preparation and dilution adheres to best practices in pharmaceutical and laboratory protocols, ensuring the safety and efficacy of the medication for its intended use.

Bleomycin Sulphate Preparation and Dissolution

The dosage given to mice in this study will be similar to that done by Gul A and colleagues (2023) [24] whereby the mice were given 50mg Bleomycin per kg of the bodyweight of the mice.

The required amount of bleomycin Sulfate will be calculated based on the average weight of the mice. For instance, if the mice weigh approximately 25 grams each, 1.25 units of bleomycin per mouse will be needed. [26]

For a batch of 10 mice, this would be 12.5 units.

The appropriate amount of bleomycin sulfate will be weighed using an analytical balance and dissolved in sterile PBS, the volume of the solution should be approximately 100µl. This solution will be mixed thoroughly by gently swirling the solution. This will be re-filtered through a 0.22-micron filter to ensure sterility and stored in labeled vials at 4°C, ensuring that the labels include concentration, date of preparation, and any relevant handling instructions. [27]

Daily Schedule for the Seven-Day Experimental Protocol

Day 0: Baseline Measurements and Initial Treatment

Prior to the 7-day schedule, 2 separate groups of mice will be used for baseline measurements, one group will be given MT treatment, while the other group will be kept in sterile air. The same BAL fluid and lung analysis will take place to provide baseline measurements of GSH and p53 gene expression levels and to control for background. As well, assessment of overall health and baseline conditions of the mice, including weight, activity levels, and any signs of distress will take place [26]. An environmental check will occur and ensure the temperature-controlled environment (20-25°C) is stable and there is unrestricted access to food and water [26].

Day 1: Baseline Measurements and Initial Treatment

1. Mice care
   - Health Check: Assessment of overall health and baseline conditions of the mice, including weight, activity levels, and any signs of distress [26].
   - Environmental Check: Ensure the temperature-controlled environment (20-25°C) is stable and there is unrestricted access to food and water [26].

Day 1 to Day 6: Continued Treatment and Monitoring

1. Daily Health Checks
   - Monitor each mouse for general health, weight, and signs of stress or adverse reactions and record any observations related to behavior, feeding, and grooming [26].
2. Anesthesia and PBS/Bleomycin Administration
   - Preparation: Set up anesthesia drop jar and prepare sterile PBS and bleomycin solutions.
   - Anesthesia Induction: The method includes dropping the mouse into the drop jar on an impermeable mesh under which a cotton gauze is dampened and saturated with 1-2 mL of isoflurane. The mouse will be kept in the jar for 30-60 seconds until unconsciousness is observed. the mouse should exhibit signs of anesthesia, such as reduced movement and lack of response to gentle stimuli [28] . Once fully anesthetized, the mouse will be removed from the jar to prevent prolonged exposure to isoflurane as that can be harmful [26].
   - Injection: Tracheal injection of either PBS (group 1 and 2) or bleomycin sulfate (group 3 and 4) following sterile techniques. Mice will then be placed on a procedure board in a supine position. The mouse's neck and chest area will be wiped with 75% ethanol to reduce the risk of infection. An injection containing either of the solutions will be carefully inserted into the trachea with care to prevent injury. Once the mouse gains consciousness, it will be observed for any respiratory ailments caused by the incision. The mouse will be excluded from the data analysis if there are any ailments. The mice will be injected using these solutions at 8:00 AM EDT [24].
3. Molecular Hydrogen Administration
   - MH Injection: Molecular hydrogen (MH) will be dissolved in distilled water to reach a concentration of 2.5 parts per million (ppm). MH will be administered using the same procedure as PBS/Bleomycin administration. In the experimental design, Group 2 and Group 4 will receive Molecular Hydrogen Treatment (MHT) immediately after daily induction of PBS or bleomycin for seven consecutive days.
4. Post-Injection Monitoring
   - Place mice in recovery cages and monitor until they regain consciousness and observe for any immediate

adverse reactions or signs of respiratory distress [26].

5. Bronchoalveolar Lavage (BAL) Fluid Collection at End of Day
   - The mice will be anesthetized, using the same procedure explained for day 1 [28] and BAL fluid collection will be performed as described in the protocol by Van Hoecke and colleagues (2017) [29]. However, instead of using EDTA, sterile PBS solution will be used since that is the standardized solution that the mice will be injected with throughout this study. A blunt ended needle will be inserted into the trachea and secured with surgical tape, a syringe will be filled with 1.5ml PBS, and infused into the trachea using the inserted needle, then the fluid will be aspirated back into the syringe, this infusion and aspiration will be repeated 3 more times to yield BAL fluid. The BAL fluid will be collected in a microfuge tube and kept on ice, which will then be stored at -80°C for subsequent analysis [29].
6. Post-Surgical Care
   - Since the mice will not be euthanized, the mice will be in recovery cages and monitored until they regain consciousness and observe for any immediate adverse reactions or signs of respiratory distress [26].

Day 7: Final Treatment and Sample Collection

1. Morning
   - Health Check: Conduct a final assessment of the mice, noting any significant changes in health or behavior [26].
2. Final Anesthesia and MH Administration
   - Follow the same procedures for anesthesia induction and MH administration as on previous days.
3. Sample Collection
   - Bronchoalveolar Lavage (BAL) Fluid Collection: The mice will be anesthetized and BAL fluid collection will be performed using the same procedure explained for day 1-7 [28]. The mice will be completely euthanized before collecting BAL fluid in contrast to days 1-6, using gradual fill method whereby the mice will be put out in small 7.5" x 11.75" x 5" cages and using CO_2 gas cylinders [30].
   - Lung Tissue Collection: A thoracotomy will be performed on the euthanized mice by carefully dissecting through the subcutaneous tissue and muscle layers using fine sterile scissors and carefully extracting them using sterile forceps and scissors. The extracted lungs will be stored in formaldehyde (10%) for fixation ensuring complete immersion and proper labeling of samples [26]. The euthanized mice will then be carefully handled by placing them in leakproof bags, and labeling with date and researcher's name and [31]. Lung tissues will be placed in formaldehyde for fixation.

Analysis Post Day 7

1. BAL Fluid Analysis
   - BAL fluid stored in microfuge tubes will first be thawed then centrifuged at 300g for 10 minutes at 4°C to pellet the cells. The supernatant will be collected and used for ROS analysis. The cell pellet will be resuspended in PBS for p53 activity analysis and Gene expression analysis.
   - p53 Activity and Gene Expression Analysis: This will be done by quantitative real-time polymerase chain reaction (qRT-PCR). The RNA will be extracted using QIAGEN RNeasy Mini Kit [32]. The resuspended cell pellet will firstly be disrupted adding buffer RLT from the kit, and adding 10 µL of β-mercaptoethanol per 1 mL of buffer RLT as per procedure for using the mini kit. Then, the cell pellet will be homogenized using a vortex. The lysate will then be transferred to a QIAdshredder spin column and centrifuged at 14000 g for 2 minutes. 350 µL of 70% ethanol will be added and mixed well by pipetting. RNA Binding will then be done by transferring the sample to an RNeasy spin column and centrifuged for 15 seconds at 8000 g. The sample will then be washed with RW1 and RPE buffers by adding 700 µL of the buffer and centrifuged for 15 seconds at 8000 g. After this, the sample will be eluted by using 50µ RNase-free water directly to the spin column membrane and centrifuged for 60 seconds at 8000 g [32]. The RNA concentration will be measured using a spectrophotometer to check for purity using the A260/A280 ratio [34].
   - The RNA sample will then be incubated at 65°C for 5 minutes in a water bath and quickly chilled for 1 minute, this is to remove any secondary structures that will be formed on the RNA. After this, 1 µg of total RNA pellet will be mixed with 1 µL of each primer; a forward primer 5'-CAGCACATGACGGAGGTTGT-3', a reverse primer 5'-TCATCCAAATACTCCACACGC-3', reverse transcriptase, 1 µL of dNTP mix and nuclease-free water to a final volume of 10 µL [34]. This is for cDNA synthesis. This mixture will be heated at 55°C for 20 minutes in a water bath, following this will be an incubation at 70°C for 5 minutes to inactivate the reverse transcriptase. Next is annealing to allow primers to bind by incubating at 59°C as that is based on the melting temperature of the primers [35]. Once all the steps are complete, the ΔΔCt will be calculated for each group to compare the expression level of the p53 gene [34].
   - ROS Analysis By Measurement of Total Glutathione, GSH, GSSG, GSH/GSSG ratio. (1) We

will be using HPLC as a semi-quantitative method to measure and calculate 4 factors of Glutathione; total glutathione, reduced glutathione (GSH), oxidized glutathione (GSSG), and GSH/GSSG Ratio. (2) BAL fluid supernatant samples will be deproteinized further to remove proteins that might interfere with the HPLC analysis. An equal volume of 5% metaphosphoric acid (MPA) will be added to the BAL fluid. The mixture will be incubated on ice for 5 minutes and then centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant, which contains the deproteinized glutathione, will be collected. For the HPLC analysis, reduced glutathione (GSH) will be derivatized using a derivatization reagent such as orthophthalaldehyde (OPA). The OPA reagent will be prepared according to the manufacturer's instructions. To 100 µL of the deproteinized BAL fluid, 10-20 µL of OPA reagent will be added, mixed thoroughly, and allowed to react for 5-10 minutes at room temperature. This derivatization step will enhance the detection of GSH by forming a fluorescent derivative [36]. The HPLC system will be set up with a reverse-phase C18 column and equilibrated with the mobile phase, which could be a mixture of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) [37]. Standard solutions of GSH and GSSG at known concentrations will be prepared and derivatized in the same way as the samples [38]. Samples of 20-50 µL of the derivatized BAL fluid or standard will be injected into the HPLC system. Gradient elution will separate GSH and GSSG, and the compounds will be detected with a UV spectrophotometer. For OPA derivatives, common detection wavelengths will be 340 nm for excitation and 420 nm for emission [36]. The peaks corresponding to GSH and GSSG will be integrated, and their concentrations will be calculated by comparing the peak areas to those of the standards. Total glutathione will be calculated as the sum of GSH and twice the concentration of GSSG (since each GSSG molecule is equivalent to two GSH molecules). The GSH/GSSG ratio will be determined by dividing the concentration of GSH by that of GSSG.

2. Lung Tissue Analysis
   - To assess fibrotic activity in lung tissue, a comprehensive histological staining process will be employed. First, the extracted lung tissues from euthanized mice will be fixed in 10% neutral buffered formalin for 24 hours at room temperature. The tissues will then be dehydrated through a series of ethanol solutions (70%, 95%, and 100%) for one hour each, followed by clearing in xylene for two hours to remove ethanol and make the tissues transparent. Next, the cleared tissues will be infiltrated with melted paraffin wax at 60°C for two hours to ensure complete infiltration, then embedded in paraffin blocks to solidify. Using a microtome, the paraffin-embedded tissues will be cut into thin sections (5-7 micrometres) and collected on glass slides after floating on a 40°C water bath. The sections will dry overnight at room temperature.
   - For deparaffinization and rehydration, the slides will be immersed in xylene (two changes, ten minutes each) to remove the wax, followed by rehydration through decreasing ethanol concentrations (100%, 95%, and 70%) for five minutes each, and a rinse in distilled water. The rehydrated sections will then be stained with Sirius Red dye for one hour at room temperature to selectively bind to collagen fibres, essential for detecting fibrosis, followed by rinsing in 0.5% acetic acid to remove excess dye. Optionally, the sections can be counterstained with hematoxylin for five minutes to visualize cell nuclei, with a subsequent rinse in running tap water. The stained sections will be dehydrated through increasing ethanol concentrations (70%, 95%, and 100%) for five minutes each and cleared in xylene (two changes, ten minutes each). Finally, the sections will be mounted with a coverslip using a mounting medium and allowed to dry completely.
   - For microscopic examination, the sections will be inspected under a polarizing light microscope where areas of fibrosis will appear red due to Sirius Red binding to collagen fibres. Additionally, a quantitative technique known as the Sircol Collagen Assay will measure the amount of soluble collagen in lung tissue. This involves solubilizing collagen, binding it to a dye, and measuring absorbance with a spectrophotometer. Comparing collagen content across experimental groups will help assess the extent of fibrosis. This detailed protocol ensures accurate histological assessment and quantification of fibrosis in lung tissue samples.

Table 1. Seven-Day Schedule Framework

Day	Time	Task	Description
Day 0	All Day	Baseline Measurements and Initial Treatment	Conduct initial health checks, verify environmental conditions, and prepare for anesthesia and treatment.
Day 1	8:00 AM EDT	Health Check	Assess health of mice (weight, activity, distress signs) [26].

Day	Time	Task	Description
	8:30 AM EDT	Environmental Check	Ensure temperature (20-25°C) and that mice have access to food and water [26].
	9:00 AM EDT	Anesthesia Preparation	Set up an anesthesia drop jar, prepare PBS and bleomycin solutions.
	9:15 AM EDT	Anesthesia Induction	Anesthetize mice using isoflurane in a drop jar [28].
	9:45 AM EDT	Tracheal Injection	Inject PBS or bleomycin into trachea; observe for respiratory issues [24].
	10:30 AM EDT	Molecular Hydrogen Administration	Administer 2.5 ppm MH for Groups 2 and 4.
	11:00 AM EDT	Post-Injection Monitoring	Monitor mice in recovery cages for adverse reactions [26].
	Afternoon	Bronchoalveolar Lavage (BAL) Fluid Collection	Collect BAL fluid for analysis [29].
	3:00 PM EDT	Post-Surgical Care	Monitor mice for signs of distress or adverse reactions [26].
Day 2 to Day 6 (each step daily)	Morning	Daily Health Checks	Monitor and record general health, weight, and behaviour [26].
	8:00 AM EDT	Anesthesia and PBS/Bleomycin Administration	Repeat anesthesia and PBS/bleomycin injection procedures [24].
	8:30 AM EDT	Molecular Hydrogen Administration	Administer 2.5 ppm MH for Groups 2 and 4 [24].
	9:00 AM EDT	Post-Injection Monitoring	Place mice in recovery cages and monitor for adverse effects [26].
	Afternoon	Bronchoalveolar Lavage (BAL) Fluid Collection	Perform BAL fluid collection for analysis [29].
	3:00 PM EDT	Post-Surgical Care	Observe mice for immediate reactions and ensure well-being [26].
Day 7	Morning	Health Check	Perform a final health assessment of the mice [26].
	8:00 AM EDT	Final Anesthesia and MH Administration	Administer anesthesia and 2.5 ppm MH for final BAL fluid collection [28].
	8:30 AM EDT	Sample Collection	Collect BAL fluid and euthanize mice for lung tissue collection [30].
	9:00 AM EDT	Lung Tissue Collection	Perform thoracotomy to extract lung tissues; store in formaldehyde [26].
	10:00 AM EDT	Post-Surgical Care	Handle and prepare euthanized mice for storage [31].
Analysis Post Day 7		BAL Fluid Analysis	Thaw, centrifuge, and analyze BAL fluid for ROS, p53 activity, and gene expression [32] [34].
		ROS Analysis	Measure total glutathione, GSH, GSSG, and GSH/GSSG ratio using HPLC [36] [37].
		Lung Tissue Analysis	Perform histological staining, deparaffinization, and Sircol Collagen Assay for fibrosis assessment [26].

Statistical Analysis

To analyze the data obtained from the study, a comprehensive statistical analysis will be required to determine the significance of the observed effects of Molecular Hydrogen Therapy (MHT) on oxidative stress and fibrotic activity. Initially, descriptive statistics, including means and standard deviations, will be calculated for each group (experimental and control) to summarize the data.

To compare oxidative stress markers (such as GSH levels) and p53 activity among the different groups, an Analysis of Variance (ANOVA) will be performed to identify any significant differences between groups. If ANOVA indicates significant differences, post-hoc tests such as Tukey's HSD (Honestly Significant Difference) will be used to pinpoint specific group comparisons. Additionally, a two-way ANOVA may be applied to examine the interaction effects between bleomycin treatment and MHT. For the histological data, quantitative measures of fibrosis will be analyzed using t-tests or Mann-Whitney U tests, depending on the data distribution, to compare between the bleomycin-only and bleomycin + MHT groups. Furthermore, Pearson or Spearman correlation analyses will be employed to assess relationships between oxidative stress markers and fibrosis levels. All statistical analyses will be performed using statistical software, with a significance level set at $p < 0.05$.

Expected Results

The expected results of the proposed study investigating the therapeutic impact of Molecular Hydrogen Therapy (MHT) on oxidative stress in a murine model of Pulmonary Fibrosis induced by bleomycin administration may include reduction in oxidative stress levels. It is anticipated that the group receiving MHT following bleomycin-induced pulmonary fibrosis will exhibit lower levels of oxidative stress compared to the bleomycin-only group. This reduction in oxidative stress may be evidenced by a decrease in markers such as glutathione (GSH) oxidation and reactive oxygen species (ROS) levels in bronchoalveolar lavage (BAL) fluid.

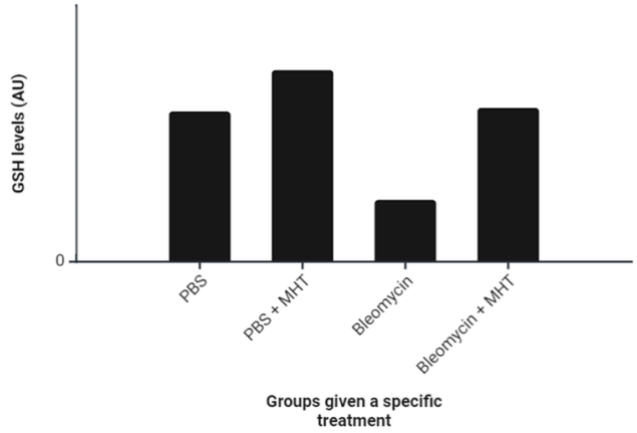

Figure 2. The anticipated graph elaborates the comparison in GSH levels between each group, with Group 1 having baseline GSH levels, Group 2 having higher than baseline levels, Group 3 having lower than baseline GSH levels, and Group 4 having comparable levels to Group 1. Figure made with BioRender.

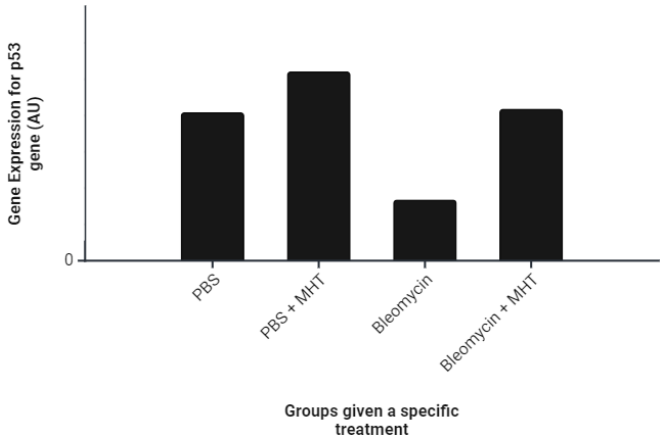

Figure 3. The anticipated graph elaborates on the comparison in expression levels of p53 gene between each group, with Group 1 having baseline expression, Group 2 having higher than baseline levels, Group 3 having lower than baseline GSH levels, and Group 4 having comparable levels to Group 1. Figure made with BioRender.

Comparison Across Groups

Group 1 (PBS) is expected to show baseline p53 activity. Group 2 (PBS + MHT) is expected to show similar or slightly elevated p53 activity compared to Group 1, due to the antioxidative effects of MHT. Group 3 (Bleomycin) is expected to show suppressed p53 activity due to high oxidative stress. Group 4 (Bleomycin + MHT) is expected to show higher p53 activity compared to Group 3, indicating the protective effect of MHT against oxidative stress-induced p53 suppression.

Histology Results

For Group 1 (PBS Control, n=10), lung tissue sections are expected to show normal lung architecture with no significant signs of fibrosis. Using Sirius Red Staining, we expect minimal to no red staining, indicating a lack of collagen deposition. For Group 2 (PBS + MHT Control), we expect similar histology result to Group 1, where lung tissues in this group should display normal architecture without fibrosis. The administration of Molecular Hydrogen Therapy (MHT) is not expected to induce fibrosis. Using Sirius Red Staining, we expect minimal to no red staining, indicating a lack of collagen deposition. For Group 3 (Bleomycin), lung tissue sections are expected to show significant fibrotic changes, including thickened alveolar walls, infiltration of inflammatory cells, and extensive collagen deposition. This reflects the damage and remodelling induced by bleomycin. Using Sirius Red Staining, we expect prominent red staining throughout the lung tissue, indicating extensive collagen deposition and fibrosis. For Group 4 (Bleomycin + MHT), lung tissues are expected to show some level of fibrosis due to bleomycin exposure, but the extent of fibrotic changes should be reduced compared to the bleomycin-only group. MHT is hypothesized to mitigate the oxidative stress and inflammatory response, leading to less collagen deposition. Using Sirius Red Staining, we expect moderate red staining, less intense than the Bleomycin group, indicating reduced collagen deposition and fibrosis.

Visual Representations

The PBS control group is expected to show normal lung architecture. Alveoli should appear open and clear, with thin alveolar walls and minimal inflammatory cells. Sirius Red staining will show minimal to no red staining, indicating no significant collagen deposition or fibrosis. Similar to the PBS control group, this group should display normal lung tissue architecture. The alveoli should remain open and clear, with thin walls and minimal inflammation. Sirius Red staining will show minimal to no red staining, confirming no collagen deposition. This demonstrates that MHT alone does not impact lung tissue structure. The bleomycin group is expected to show significant fibrotic changes. Alveoli will likely be distorted with thickened walls and increased inflammatory cells. Sirius Red staining will reveal extensive red staining, indicating high levels of collagen deposition and fibrosis, typical of bleomycin-induced lung injury. In this group, some fibrotic changes are expected, but less severe than in the bleomycin-only group. Alveoli should be less distorted and walls less thickened. Reduced inflammation is anticipated. Sirius Red staining should show moderate red staining, indicating reduced collagen deposition and fibrosis due to MHT's protective effects.

Discussion

The primary objective of this study was to investigate the potential therapeutic efficacy of Molecular Hydrogen Therapy (MHT) in mitigating oxidative stress and fibrosis in a murine model of bleomycin-induced idiopathic pulmonary fibrosis (B-IPF). To achieve this goal, we will employ a comprehensive experimental approach that would involve the induction of IPF in mice through bleomycin administration, followed by the assessment of ROS levels by measuring GSH and oxidized glutathione GSSG and levels, gene expression of p53 gene, and p53 activity in response to MHT treatment. However, it is crucial to note that the results discussed herein are anticipated based on existing literature and hypothesized outcomes, not from actual data collected in this study.

Our experimental design includes four mice groups: two subjected to bleomycin injections to induce IPF and two control groups administered phosphate-buffered saline (PBS). Within each set, one subgroup received MHT treatment, while the other was exposed to sterile air. Bronchoalveolar lavage (BAL) was utilized to quantify GSH levels before and after MHT administration, providing insights into the antioxidative effects of MHT. Additionally, RNA sequencing was employed to evaluate p53 activity across all treatment and control groups, elucidating the molecular mechanisms underlying the therapeutic effects of MHT.

Analysis of the expected results may reveal several key findings regarding the impact of MHT on oxidative stress and fibrosis in bleomycin-induced IPF as supported by previous studies which support our expected findings in Figures 2 and 3. For example, [39] found that molecular hydrogen has potent antioxidative effects, which could result in higher levels of GSH in MHT-treated mice compared to untreated controls [39]. This increase in GSH levels suggests that MHT may enhance the lung's antioxidant defenses, thereby reducing oxidative damage and protecting against fibrotic tissue remodeling, consistent with findings by [21] who reported similar antioxidative benefits of MHT [21]. This is also reflected in our anticipated findings in Figure 2 where we hypothesize an expected increase in GSH levels for groups under MHT.

Furthermore, assessment of p53 activity should reveal that MHT treatment will result in a more pronounced activation of p53 signalling pathways than untreated controls. Activation of p53 is known to promote cellular repair mechanisms and apoptosis of damaged cells, thereby

limiting tissue injury and fibrotic remodeling [40]. The observed enhancement of p53 activity in response to MHT suggests that this therapy may exert its beneficial effects by modulating p53-mediated cellular responses to oxidative stress and DNA damage [41]. While we do know that persistent activation of p53 can lead to damaged signalling and inflammation [10] we hypothesize that p53 gene expression should increase due to its nature of being a suppressor gene as shown in Figure 3. However, this gene should be further studied in fibrosis to understand its mechanism more deeply.

These anticipated findings may support the potential utility of MHT as a therapeutic intervention for IPF by targeting key molecular pathways involved in oxidative stress and fibrosis. By restoring the balance of harmful molecules in the body and enhancing the body's ability to repair lung cells, MHT holds promise for attenuating lung injury and preserving lung function in patients with IPF. The broader implications of this study for clinical practice could be profound. If Molecular Hydrogen Therapy (MHT) proves effective in reducing oxidative stress and fibrosis in bleomycin-induced idiopathic pulmonary fibrosis (IPF), it could revolutionize the management of not only IPF but other oxidative stress-mediated lung diseases as well. Given the limited treatment options currently available for IPF, MHT offers a novel therapeutic approach that targets the underlying mechanisms of oxidative damage and fibrotic remodelling. Clinical trials would be necessary to validate these findings in human patients, potentially leading to new standards of care that incorporate MHT alongside existing treatments such as pirfenidone and nintedanib [40, 41]. Furthermore, understanding the molecular pathways modulated by MHT, such as the p53 signaling pathway, could pave the way for combination therapies that enhance the overall therapeutic efficacy [42]. This study also highlights the importance of monitoring oxidative stress markers and genetic regulators in managing pulmonary fibrosis, offering a more personalized approach to treatment [10]. Additionally, the potential off-target effects and long-term safety of MHT need thorough investigation to ensure its clinical applicability and patient safety [44]. Therefore, integrating MHT into clinical practice could significantly improve patient outcomes and quality of life by providing a targeted, effective treatment for IPF and potentially other related diseases.

While functional assessments such as lung function tests were not explicitly outlined in our methods, they remain crucial for evaluating the efficacy of therapeutic interventions in pulmonary fibrosis. Studies in similar contexts have demonstrated the importance of assessing lung function parameters to comprehensively understand the impact of treatment on respiratory health. For instance, in a study by [42], the authors evaluated the effects of molecular hydrogen on pulmonary hypertension in rats induced by monocrotaline (MCT). They observed significant improvements in hemodynamics and pulmonary vascular remodeling following treatment with hydrogen-saturated water. Functional assessments, including lung compliance, airway resistance, and gas exchange efficiency, were likely instrumental in assessing these improvements [43]. The findings from [42] support our hypothesis that MHT could lead to improvements in lung function parameters in our murine model of bleomycin-induced pulmonary fibrosis. Specifically, MHT may enhance lung compliance, reduce airway resistance, and improve gas exchange efficiency, ultimately contributing to better respiratory function in fibrotic lungs.

While the primary focus is on mitigating oxidative stress associated with fibrosis, the study may uncover unanticipated physiological responses or adverse reactions to MHT that were not previously recognized. Moreover, variations in treatment responses within different subgroups of bleomycin-injected mice could challenge the generalizability of the findings, highlighting the importance of considering individual differences in therapeutic outcomes. Similarly, unexpected differences in treatment efficacy between animal models and human populations may arise, necessitating a nuanced approach to translating research findings into clinical practice. Furthermore, exploring alternative molecular pathways beyond the known role of ROS and p53 modulation could reveal novel insights into the pathogenesis of IPF and potential therapeutic targets. These unforeseen mechanisms or biomarkers could significantly impact our understanding of the disease and guide the development of more effective treatment strategies. Additionally, the study may uncover unforeseen interactions between MHT and bleomycin, or even negative effects of MHT itself, underscoring the importance of comprehensive evaluation and monitoring in clinical settings. Despite the potential for unexpected findings, this research holds promise for advancing our understanding of IPF and exploring innovative treatment approaches.

Although our study aims to explore the impact of MHT on oxidative stress and p53 activity, it does not address potential off-target effects or long-term safety considerations associated with MHT administration. As mentioned earlier, this protocol is based on animal models; therefore, future directions for research in this area could involve conducting additional preclinical studies to validate the efficacy and safety of MHT in bleomycin-induced pulmonary fibrosis, as well as exploring alternative treatment modalities targeting oxidative stress pathways or p53 signalling. Moreover, clinical trials are warranted to assess the translational potential of MHT and other therapeutic approaches in human patients with bleomycin-induced pulmonary fibrosis, with a focus on optimizing treatment strategies to improve patient outcomes and quality of life. Additionally, further investigation into the underlying mechanisms of bleomycin-induced oxidative stress and fibrosis could uncover novel therapeutic targets

for the development of more effective treatments for this debilitating condition.

Conclusion

This study aims to investigate the therapeutic efficacy of Molecular Hydrogen Therapy (MHT) in mitigating oxidative stress and fibrosis in a murine model of bleomycin-induced idiopathic pulmonary fibrosis (B-IPF). Our experimental design involved inducing IPF in mice through bleomycin administration and subsequently assessing the effects of MHT on oxidative stress markers, p53 gene expression, and p53 activity. The anticipated results, grounded in existing literature, suggest that MHT has the potential to significantly reduce oxidative stress and fibrosis.

Our anticipated findings support the hypothesis that MHT enhances antioxidant defenses, evidenced by increased GSH levels and reduced oxidative damage in MHT-treated mice. Additionally, MHT is expected to modulate p53 activity, promoting cellular repair mechanisms and apoptosis of damaged cells, thereby limiting tissue injury and fibrotic remodeling. These anticipated results underscore the potential utility of MHT as a therapeutic intervention for IPF by targeting key molecular pathways involved in oxidative stress and fibrosis.

Moreover, the broader implications of this study for clinical practice are profound. If validated, MHT could revolutionize the management of IPF and other oxidative stress-mediated lung diseases. The potential for MHT to be integrated into standard care, alongside existing treatments such as pirfenidone and nintedanib, could significantly improve patient outcomes and quality of life. Additionally, this study highlights the importance of monitoring oxidative stress markers and genetic regulators in managing pulmonary fibrosis, offering a more personalized approach to treatment.

However, our study also emphasizes the need for comprehensive evaluation and monitoring of potential off-target effects and long-term safety of MHT. Further preclinical studies and clinical trials are warranted to validate these findings and optimize treatment strategies. Understanding the underlying mechanisms of bleomycin-induced oxidative stress and fibrosis could uncover novel therapeutic targets, guiding the development of more effective treatments for this debilitating condition.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Ethics Approval and/or Participant Consent

This research proposal did not require a review by the research ethics board of the institution the authors belong to as there is no such requirement for research proposals. This research protocol will need to be reviewed by the Institutional Animal Care Committee (IACC) to ensure compliance with Canada Council on Animal Care (CCAC) guidelines and ethical principles due to the use of animal models, including the principles of the Three Rs: Replacement, Reduction, and Refinement.

Authors' Contributions

KB: Involved in conceptualization, methodology design, designing specific experimental procedures (animal models and laboratory techniques), data analysis, and final approval of the manuscript version to be published.
TR: Involved in conceptualization, literature review, methodology design, data analysis and interpretation, and final approval of the manuscript version to be published.
ZA: Involved in conceptualization, literature review, methodology design, data analysis and interpretation, and final approval of the manuscript version to be published.

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