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# PHOTOBIOMODULATION THERAPY REDUCES HYPOXIA-INDUCED LUNG INJURY IN SPRAGUE DAWLEY RATS UNDER HYPOBARIC CONDITIONS

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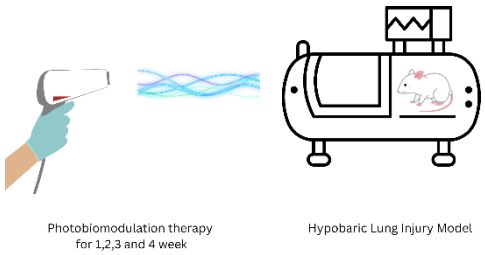
## Graphical abstract




## Abstract

Hypoxia in hypobaric conditions refers to the reduced availability of oxygen due to decreased barometric pressure at high altitudes. As altitude increases, atmospheric pressure decreases, leading to a lower partial pressure of oxygen (PO2) and reduced oxygen saturation in blood and tissues. Photobiomodulation therapy (PBMT) has emerged as a potential adjunctive treatment for lung injury, offering non-invasive and promising benefits. PBMT has shown effectiveness in modulating inflammatory responses, reducing oxidative stress, promoting tissue repair, and improving respiratory function in conditions such as acute respiratory distress syndrome (ARDS), pneumonia, and pulmonary oedema. In this study, thirty 8-week-old male Sprague Dawley rats were divided into six groups: (i) normal control (Normal), no hypobaric exposure or treatment; (ii) negative control (Negative Control), hypobaric treatment without PBMT; (iii) one-week PBMT (PBMT1); (iv) two-week PBMT (PBMT2); (v) three-week PBMT (PBMT3); and (vi) four-week PBMT (PBMT4). Hypobaric exposure was performed weekly for 28 days at the Lakespra Facility (Indonesian Military Air Force, Jakarta, Indonesia) at an altitude of 25,000 feet for five minutes. PBMT was administered every two days with a stimulation energy dose of 0.4 Joule (2.037 J/cm²), totaling 8.15 J/cm² per day. The results indicated that PBMT significantly reduced a lung oedema index, lung injury severity, and expression of IL6, CD73, and adenosine, though it did not consistently reduce HIF-1 expression in lung tissue. In conclusion, PBMT effectively prevented lung injury induced by hypobaric hypoxia.

*Keywords*: Lung hypoxia, hypobaric, photobiomodulation, 650 nm, histopathology, IL6

## 1.0 INTRODUCTION

Hypoxia, a state of reduced oxygen levels in the body's tissues, can result from several lung injuries and oxidative damage caused by the hypoxia pathway [1][2]. Hypoxia is a prominent characteristic of acute respiratory distress syndrome (ARDS). It is mainly produced by a mismatch between ventilation and perfusion, intrapulmonary shunting, and limited diffusion due to disruption to the alveolar-capillary membrane [3]. Hypoxia in pneumonia can occur due to several factors, including ventilation-perfusion mismatch, mitochondrial dysfunction, alveolar consolidation, and decreased gas exchange caused by inflammatory exudates in the alveoli [4].

Hypoxia under hypobaric situations is when there is a decrease in the amount of oxygen in the air due to the lower barometric pressure at high elevations. As elevation rises, the atmospheric pressure declines, decreasing the partial pressure of oxygen (PO2) and ultimately causing a decrease in oxygen saturation in the blood and tissues [5]. Exposure to high altitudes might hinder the physiological adaptation process called acclimatization, especially in patients with lung damage [6]. Conditions such as low oxygen levels in the blood (hypoxemia), inflammation, and decreased function of the blood vessels in the lungs can make it difficult for the body to adapt to changes in altitude. This increases the likelihood of developing altitude-related disorders such as high-altitude pulmonary edema (HAPE) and high-altitude cerebral oedema (HACE) [7].

Photobiomodulation therapy (PBMT) has emerged as a potential additional treatment for lung injury, providing non-invasive and promising therapeutic advantages. Photobiomodulation therapy (PBMT) has demonstrated efficacy in regulating inflammatory reactions, diminishing oxidative stress, facilitating tissue regeneration, and enhancing respiratory capacity in lung conditions such as ARDS, pneumonia, and pulmonary oedema [8], [9], [10]. Utilizing the PBMT approach, low-level laser therapy can decrease acute lung inflammation in a model of pulmonary and extrapulmonary LPS-induced ARDS [11]. PBMT has demonstrated efficacy in the treatment of sepsis-induced lung injury [12] and, intestinal ischemia and reperfusion-induced lung injury [13].

The low efficacy of traditional oxygen treatment in extreme situations makes treating hypoxia-induced lung damage in clinical settings difficult, particularly when combined with oxidative stress and inflammation. Ventilator-induced lung injury (VILI), while typically required, may result in additional lung damage when using mechanical ventilation. These side effects highlight the need for supplemental therapies like photobiomodulation therapy (PBMT), a non-invasive option that targets cellular processes to improve oxygenation, decrease inflammation, and promote tissue repair. PBMT is a promising treatment for patients with hypoxic lung injuries [11].

PBMT therapy uses irradiation to activate the tissues by stimulating cellular photoreceptors, including cytochrome c oxidase, porphyrins, and light-sensitive ion channels. As a result, electron transport, mitochondrial membrane potential, and adenosine triphosphate (ATP) synthesis are elevated, leading to a significant genetic reaction focused on inflammation, proliferation, and repair [8-10]. Using 660 nm red visible light has been shown to enhance NF-KappaB and AP-1 cell signaling [15].

## 2.0 METHODOLOGY

### Ethical Approval

The studies were conducted at the Indonesian Air Force Institute of Aviation Medicine (Lembaga Kesehatan Penerbangan dan Ruang Angkasa Lakespra) and the iRATco Veterinary Laboratory in Bogor. The Faculty of Medicine, Public Health and Nursing Universitas Gadjah Mada scientific ethics committee accepted all procedures (Ref. No.: KE/FK/1636/EC/2022).

### Animals

Thirty 8-week-old male Sprague Dawley rats were acquired from iRATco Veterinary Laboratory Services (Indonesia) and categorized into six groups:

- Normal control group (Normal): Animals not exposed to hypobaric conditions and receiving no therapy.
- The negative control group (Negative Control) consists of animals that receive hypo-baric therapy but do not undergo Photobiomodulation Therapy (PBMT).
- PBMT1 refers to a group of animals who received hypobaric treatment plus one week of PBMT.
- PBMT2: A group of animals with hypobaric treatment and two weeks of PBMT.
- PBMT3: A group of animals with hypobaric treatment and three weeks of PBMT.
- The four-week PBMT group (PBMT4) consisted of animals who received hypobaric treatment and underwent four weeks of PBMT.

### Hypobaric Exposure and PBMT

The animals were exposed to hypobaric conditions every seven days for a total of 28 days at the Lakespra Facility, which is located in Jakarta, Indonesia, and is operated by the Indonesian Military Air Force. The altitude during the exposure was set at 25,000 feet, and each session lasted for five minutes, as previously explained, modification Kumar [16]. Control measures were in place to ensure consistent environmental conditions during hypobaric exposure, including maintaining stable temperature and humidity levels throughout the sessions. This helped minimize variability in the experimental conditions. The PBMT energy calculations can be summarized as follows: each session involved applying 0.4 Joules over an area of 0.1963 $cm^2$, resulting in an energy density of 2.037 $J/cm^2$. With treatments

administered every other day, the cumulative daily dose was 8.15 J/cm², making the calculations clear and easy to follow.

### Euthanasia and Sample Preparation

Following the 28-day experiment, the animals were terminated with Ketamine (100 mg/KgBW) and Xylazine (5 mg/KgBW). The lung tissues were preserved in a 10% Neutral Buffered Formalin (NBF) solution to prepare them for analysis under a microscope.

### Lung Edema Index

The lung oedema index was determined by measuring the weight of the lungs in their wet and dried states (after being dried at 60°C for 4 hours). The formula was used to determine the lung oedema index.

Lung edema index = $(gr\ wet\ -\ gr\ dry) \div gr\ dry$

The variable "gr wet" represents the weight of the lung lobe in its fresh state, whereas "gr dry" represents the weight of the lung lobe after it has been dried.

### Lung Macroscopy Score

The evaluation of lung macroscopy was conducted using the following scoring system: Score 0: Lung in a normal state; Score 1: Slight inflammation and paleness; Score 2: There is a moderate amount of oedema and a small amount of bleeding; Score 3: There is a severe amount of bleeding, and the person is experiencing collapse.

### Type-2 Pneumocyte Proportion

The lung tissue was preserved in NBF and treated using the standard paraffin embedding technique. The paraffin blocks were cut into sections with a thickness of 5 µm and then stained using Haematoxylin and Eosin (HE). The ratio of type II to type I epithelial alveolar cells was quantified in five fields of view per lung at a magnification of 400X. The fraction of type 2 pneumocytes was determined using the following formula:

T2 pneumocyte % = $(T2\ /\ T1) \times 100$

T1 represents the overall quantity of type-1 pneumocytes present in the alveolus, while T2 represents the overall quantity of type-2 pneumocytes in the same alveolus.

### Protein Expression in the Lung by Immuno- labelling

Identical paraffin-embedded lung tissue blocks were divided into sections for examination using immunohistochemistry (IHC). The sections underwent deparaffinization using xylene, rehydration using various ethanol strengths and finally brought to distilled water (DW). The antigen retrieval process was carried out by subjecting the sample to a Citrate Buffer solution at a temperature of 121°C for 10 minutes. This was followed by rinsing the sample with PBS. The activity of endogenous peroxidase was inhibited by treating it with a solution of 3% H2O2 in methanol for 30 minutes at room temperature. The protein block was conducted utilizing a mouse and Rabbit-specific HRP/DAB (ABC) detection kit (catalog number ab64264, Abcam, United Kingdom). Antibodies targeting IL-6 (catalog number ab6672, Abcam, UK), CD73, adenosine, HIF-1, and ROS were left to incubate overnight at a temperature of 4°C. The staining was seen via diaminobenzidine and observed through a microscope. The IHC figures were examined to measure the percentage of IL-6 expression in the lung using ImageJ software. Identical techniques were employed to visualize mitochondria using FITC fluorescent labeling.

### Statistical Analyses

The data distribution was assessed using the Shapiro-Wilk test, and the equality of variances was determined using the Bartlett test. Based on the specific attributes of the data, ANOVA with post hoc Tukey-HSD or paired Wilcoxon tests were chosen. The analyses were conducted using version 3.5.1 of the R software, found at www.r-project.org. Scoring charts, boxplots, and correlation data were visualized using R software.

## 3.0 RESULTS AND DISCUSSION

### Lung Oedema Index

The lung oedema index showed a substantial decrease in all groups treated with PBMT compared to the negative control group. There was no notable disparity in oedema levels across the PBMT1, PBMT2, PBMT3, and PBMT4 groups (Figure 1).

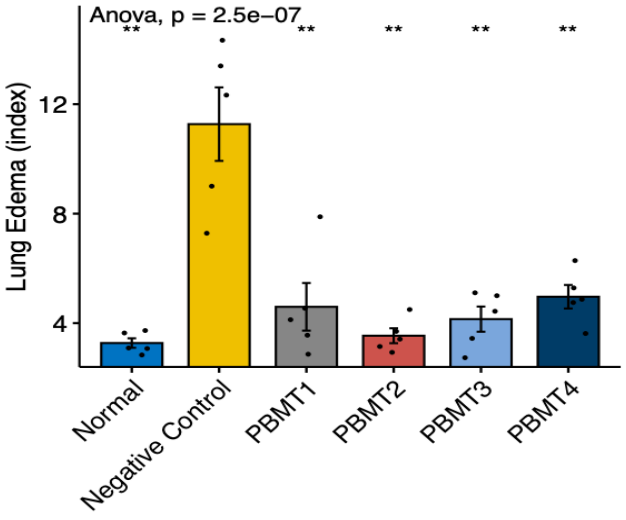



**Figure 1** The lung oedema index for all PBMT-treated groups (PBMT1, PBMT2, PBMT3, and PBMT4) shows a significant reduction compared to the negative control group. This reduction highlights PBMT's effectiveness in minimizing fluid accumulation in lung tissues under hypobaric conditions, demonstrating its therapeutic potential in managing pulmonary injuries. No significant differences were observed between the various PBMT treatment groups

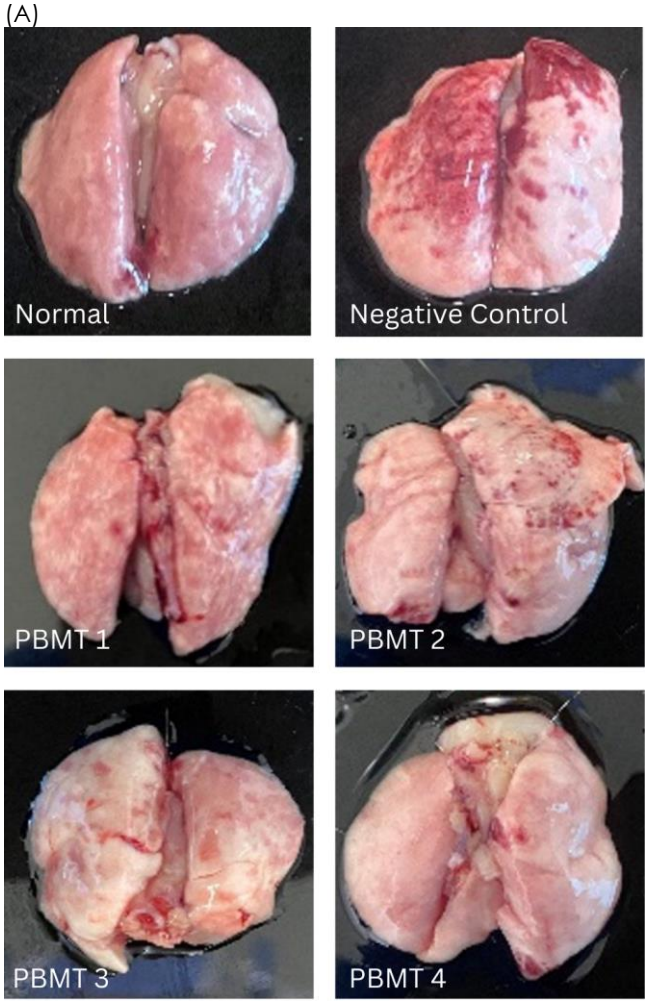
The decrease in lung oedema indicates that PBMT has a therapeutic impact in lowering the buildup of fluid in lung tissues, which is crucial for managing pulmonary injuries.

## Lung Lesion Score

The negative control group exhibited the most severe lung damage during the gross pathology assessment, characterized by extensive bleeding throughout the lung lobes. Additional bleeding was observed in the PBMT1 and PBMT2 groups, although it was less severe. No hemorrhage was seen in the PBMT3 and PBMT4 groups, suggesting that PBMT can reduce severe lung injury (Figure 2A).

The lung lesion scoring data revealed that the negative control group exhibited the highest score, indicating a significant degree of lung injury. The PBMT1 and PBMT2 groups exhibited lower scores than the negative control group, suggesting a moderate enhancement. The PBMT3 and PBMT4 groups exhibited the lowest ratings, indicating a substantial decrease in the degree of lung injury (Figure 2B).

(A)




(B)

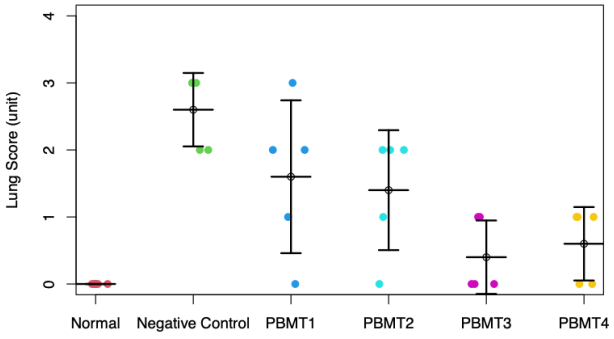



**Figure 2** (A) Macroscopic analysis of lung tissue shows that the negative control group experienced extensive bleeding across lung lobes, while PBMT1 and PBMT2 groups exhibited less severe bleeding. The PBMT3 and PBMT4 groups had no visible hemorrhaging, indicating the protective effect of PBMT in reducing severe lung injury. (B) Lung lesion score data reveals that the negative control group had the highest lung injury score, with PBMT1 and PBMT2 showing moderate improvement. PBMT3 and PBMT4 had the lowest scores, indicating a significant reduction in lung injury severity

## Type-2 Pneumocyte Proportion

Pneumocyte type 1 and 2 cells proliferatively respond to hypoxia and laser therapy. The percentage of type-2 pneumocytes showed a substantial rise in all PBMT groups compared to the negative control group (Figure 3). This rise indicates that PBMT stimulates the growth of type-2 pneumocytes, vital for the restoration of alveoli and the synthesis of surfactant, which is critical for sustaining lung function.

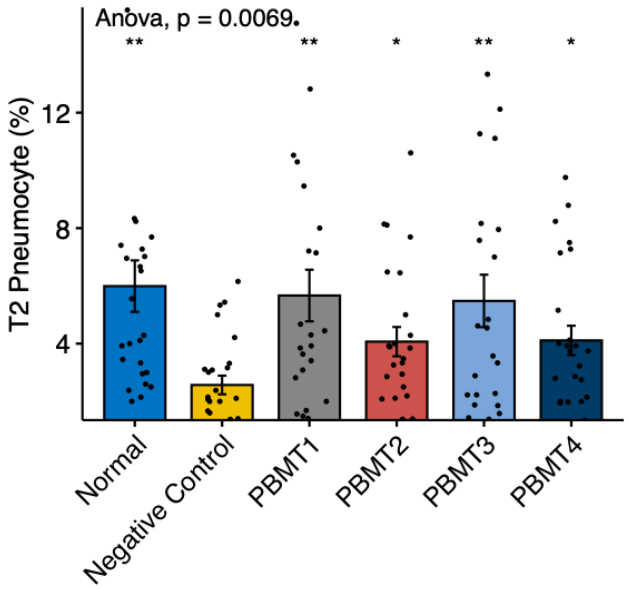



**Figure 3** Type-2 pneumocyte percentage data indicate that all PBMT groups effectively enhance type-2 pneumocyte proportion

From a histological perspective, the groups treated with PBMT showed broader alveolar spaces than the negative control group. The negative control group experienced alveolar collapse and inflammation, while the PBMT3 and PBMT4 groups showed the most notable enhancements in alveolar space and decreased inflammation (Figure 4).

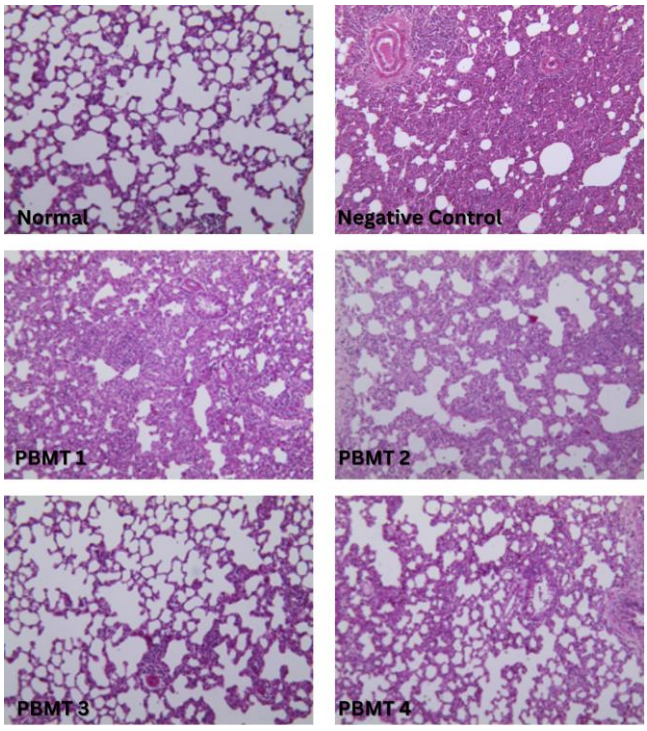



**Figure 4** Histological analysis reveals that PBMT3 and PBMT4 significantly enhance the alveolar space in comparison to PBMT1 and PBMT2

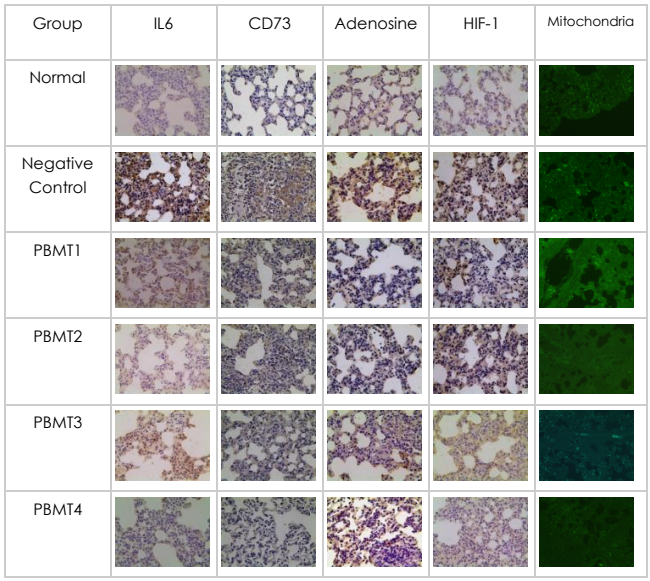
## Protein Expression in the Lung by Immunolabelling

The IL-6 expression was markedly decreased in all PBMT groups compared to the negative control group. There was no notable disparity in IL-6 expression among the PBMT1, PBMT2, PBMT3, and PBMT4 groups. Notably, there was no notable distinction between the PBMT and the normal control groups, suggesting a successful decrease of IL-6 to normal levels (Figure 5). The decrease in IL-6 indicates that PBMT can successfully alleviate the inflammatory reaction linked to hypobaric hypoxia, such as hypoxic condition in Chronic obstructive pulmonary disease COPD [17}.

CD73 expression was markedly reduced in all PBMT groups compared to the negative control group, with the PBMT4 group showing the lowest expression. CD73 participates in the anti-inflammatory pathway in purinergic signalling [18}, and its decrease further demonstrates the anti-inflammatory effects of PBMT. The mitochondrial expression data indicated a decrease in active mitochondria in all PBMT groups compared to the negative control group. PBMT1 had a greater quantity of functional mitochondria than the normal control group; however, PBMT2, PBMT3, and PBMT4 displayed notably reduced quantities compared to the normal group. These findings indicate that PBMT may assist in decreasing oxidative stress by regulating mitochondrial function [8-10].

The expression of HIF-1 did not exhibit consistent resolution throughout the PBMT groups. While the normal control group had lower HIF-1 expression, the negative control group had higher HIF-1 expression. However, none of the PBMT groups showed a significant decrease in HIF-1 expression, as seen in Figures 5A and 5B. This contradiction suggests that although PBMT has various advantageous benefits, it may not substantially influence the hypoxic response controlled by HIF-1.

(A)




(B)

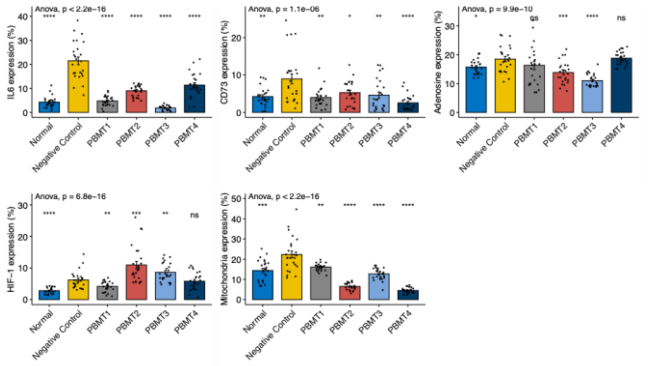



Figure 5 (A) Protein expression of IL-6, CD73, adenosine, HIF-1, and mitochondria in the lung by immunolabelling. (B) Quantitative measurement of the protein expression

## Discussion

Hypobaric hypoxia creates a low-oxygen atmosphere by reducing the air pressure and oxygen levels in the surroundings. Exposure to hypobaric hypoxia causes a decrease in the oxygen pressure in the alveoli, resulting in a reduction in lung space due to alveolar collapse and changes in lung tissue structure. Quantification of the alveolar space revealed that an increasing number of exposures led to a decrease in the area of the alveolar space. From a histological perspective, the H4 group experienced hypobaric exposure four times and exhibited the smallest alveolar space.

In a murine model of pulmonary inflammation, photobiomodulation (PBM) with a wavelength of 660 nm and an energy density of 4.5 J/cm² was applied to the skin above the right upper bronchus 15 minutes after inducing acute lung injury (ALI) [8]. The dosimetric parameters for PBM exhibited variations within the following ranges: the wavelength ranged from 660 to 830 nm, and the energy density ranged from 3 to 10 J/cm². PBM effectively decreases the number of inflammatory cells present in the alveolar space, the formation of collagen, the thickening of the interstitial tissue, and both static and dynamic pulmonary elastance. The user's text consists of the numbers 13 and 21 enclosed in square brackets. In addition, PBM showed efficacy in decreasing the levels of IL-6, a crucial mediator of inflammation, indicating its ability to regulate inflammatory responses [11].

Photobiomodulation treatment (PBMT) presumably operates via important molecular routes, such as augmenting mitochondrial function by inducing cytochrome c oxidase, which increases ATP synthesis and facilitates tissue restoration [8] [20]. By raising antioxidant enzymes like glutathione peroxidase (GPx) and superoxide dismutase (SOD), it also lessens oxidative stress and limits lung damage. By downregulating pro-inflammatory cytokines, including TNF-a and IL-6, PBMT modifies inflammatory pathways and may also impact NF-kB activation [8-10]. Furthermore, by inducing type II pneumocytes, which are essential for lung tissue regeneration, PBMT facilitates alveolar repair.

Intestinal ischemia and reperfusion (I/R) resulted in a distinct form of ALI, which mimicked the symptoms of acute respiratory distress syndrome (ARDS). Rats who underwent superior mesenteric artery blockage for 45 minutes and received photobiomodulation (at a wavelength of 660 nm and energy density of 7.5 J/cm²) had decreased inflammation in their lungs when exposed to light on the skin above the right upper bronchus for either 15 or 30 minutes [8]. This experiment employed a stimulation energy dose of 0.4 Joule, with photobiomodulation (PBM) applied to the skin at the right and left upper bronchus and liver area level for 14 days. Photobiomodulation (PBM) can impact the activity and function of pneumocytes by promoting cellular regeneration, enhancing protein synthesis and immune response, and decreasing oxidative stress. Type II pneumocytes, which exhibit enhanced responsiveness to PBM (photobiomodulation), release proinflammatory agents when exposed to oxidative stress and function as precursor cells for the regeneration of alveoli [5], [16}.

Photobiomodulation (PBM) or Low-Level Laser Therapy (LLLT) can modulate cellular signaling pathways, enhancing circumstances that deteriorate due to hypoxia. IL-6, a cytokine part of the body's inflammatory and immunological system, may impact how cells respond to PBM therapy [10], [11]. Under normal oxygen levels, photobiomodulation (PBM) can enhance the synthesis of interleukin-6 (IL-6) through biological mechanisms. This is crucial in regulating various biological activities, such as mitochondrial activity and tissue repair [8]. Earlier studies have shown that exposure to low oxygen levels (hypoxia) strongly inhibits important molecules of the body's natural defense system, such as GM-CSF, CCL2, and IL-6[8][10}. Rats exposed to hypobaric settings exhibit tiny lung lesions, accompanied by elevated levels of IL-6, indicating severe inflammation and cellular stress in the lungs [11], [17}.

According to earlier research, photobiomodulation treatment (PBMT) efficiently lowers inflammatory markers like IL-6 and encourages alveolar regeneration in lung damage models. These results are consistent with earlier findings. Variability in our data suggests that several hypoxia-response mechanisms may exist, even though numerous studies consistently show decreases in HIF-1 expression with PBMT. Corroborating our findings of elevated superoxide dismutase (SOD) levels, a comparable study also emphasizes the function of PBMT in boosting antioxidant activity. The combined results highlight the potential of PBMT as a comprehensive therapy for pulmonary disorders brought on by hypoxia [2], [18], [19].

Different cellular environments and experimental settings, such as light dose and exposure length, might cause uneven effects on HIF-1 expression. Furthermore, interactions with other signalling pathways involved in inflammation, hypoxia, and genetic heterogeneity within animal models may affect [14] HIF-1 activation. Further research on these parameters is necessary to optimize PBMT procedures in clinical applications [2], [9], 18].

This research has limitations due to animal models, Sprague Dawley rats in particular, which may not accurately represent human physiological responses to photobiomodulation treatment (PBMT) and hypobaric hypoxia. The results provide essential light on how PBMT affects lung damage. However, discrepancies between animal and human lung anatomy, immunological responses, and disease development may make applying these findings in a clinical setting challenging. Furthermore, the complexity of lung disorders due to hypoxia in humans can be different from the controlled laboratory circumstances. Therefore, further investigation is required to assess the long-term safety and effectiveness of PBMT in human

patients and better understand its potential therapeutic uses.

Future studies should apply the efficacy of PBMT in animal models to human patients with hypoxia-related lung damage. They should also investigate the best dosages, long-term safety, and efficacy in treating illnesses like pneumonia and ARDS. Studies on the impact PBMT has on important human biological pathways are necessary to validate its use as a noninvasive therapeutic in clinical practice [11].

Enhancing antioxidant activity promotes the synthesis of enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), which counteract free radicals, particularly IL-6, that are involved in inflammation, lung tissue harm, and pulmonary oedema. 660 nm red visible light can enhance NF-KappaB and AP-1 cell signaling [11], [20]. PBMT, with its ability to enhance antioxidant activity and decrease oxidative stress, can effectively suppress the generation of IL-6 and alleviate the inflammatory response that occurs after hypobaric post-hypoxia [5], [20]. Hence, PBM could be a supplementary treatment for pulmonary illness.

## 4.0 CONCLUSION

Photobiomodulation therapy has shown considerable promise as a supplementary treatment for lung injury caused by hypobaric hypoxia. The study results demonstrate that PBMT successfully reduces lung edema, severity of lung lesions, and inflammation. More precisely, PBMT was demonstrated to:

The pulmonary edema index was significantly reduced in all groups receiving PBMT compared to the negative control group.

The groups treated with PBMT3 and PBMT4 had the most significant decrease in lung lesion scores, suggesting lower lung injury severity.

Enhanced Alveolar Repair: The proportion of type-2 pneumocytes significantly increased in all PBMT groups, indicating improved repair of the alveoli.

Decrease Inflammatory Markers: The expressions of IL-6 and CD73 were considerably reduced in all PBMT groups, indicating the anti-inflammatory effects of PBMT. While PBMT did not consistently decrease HIF-1 expression, it successfully regulated other critical inflammatory pathways.

These findings indicate that PBMT can alleviate the harmful impact of hypobaric hypoxia on the lungs by diminishing inflammation, enhancing tissue regeneration, and enhancing overall lung function. The results are consistent with earlier research on the therapeutic effects of PBMT in different lung diseases, highlighting its potential for clinical use.

Nevertheless, future research should address the study's limitations, including the need for long-term follow-up and more comprehensive mechanistic studies. Additional research is necessary to examine PBMT's long-term effectiveness and to understand the underlying processes by which it produces its benefits.

To summarize, PBMT shows potential as a noninvasive treatment method for addressing lung damage caused by hypobaric hypoxia. Its capacity to decrease lung edema, improve alveolar repair, and regulate inflammatory responses makes it a valuable treatment option for hypoxia-related pulmonary diseases.

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## Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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