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Short Communication

Effects of Hyperbaric Oxygen Therapy on Lungs Histopathology of Animal Models with Chronic Obstructive Pulmonary Disease

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a respiratory or alveolar tract disorder characterized by inflammation with high morbidity and mortality rates. The increasing mortality rate among workers in certain jobs emphasizes the importance of targeted interventions to prevent COPD development. This study aimed to investigate the effects and mechanisms of hyperbaric oxygen therapy (HBOT) intervention in reducing inflammation in COPD animal models. Twenty-one female Wistar rats aged 6-8 weeks weighing between 180-200 g were used in this study. They were divided into 3 groups: G0 (negative control), G1 (COPD group without HBOT treatment), and G2 (COPD group with HBOT treatment). The HBOT dose includes breathing 98.2% O2 of 1.3 ATA 3 times at 30-minute, intervals 2 times for 5 minutes normal air, for 5 consecutive days, interspersed with 2 days of rest then 5 more consecutive days in the animal chamber. SOD and IL-1β levels in the rats' blood serum were examined using the ELISA method. Hematoxylin-eosin (HE) staining technique was used to examine the expression of inflammatory cells in the rats' lung tissue. The Games-Howell test showed a non-significant decrease in IL-1β levels ($p > 0.05$) and a significant increase in SOD enzyme activity ($p<0.05$) in G2 compared to G1. The histopathology results revealed a significant decrease in inflammatory cell expression in lung alveoli tissue ($p = 0.029$, $p<0.05$) in G2 compared to G1. The study concludes that HBOT exhibited anti-inflammatory effects in animal models of COPD and can be used as adjuvant therapy in the management of COPD.

Keywords: HBOT, COPD, IL-1*β*, SOD, Lung tissue inflammatory cells.

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a disease caused by significant exposure to dangerous particles and is influenced by various factors, including the host, leading to abnormal lung tissue.[1,2] The Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2023 defines COPD as a heterogeneous lung condition characterized by chronic respiratory symptoms (dyspnea, cough, expectoration, and/or exacerbations) due to abnormalities of the airways (bronchitis, bronchiolitis and/or emphysema) that cause persistent, often progressive airflow obstruction.[1] Inflammation, the main characteristic of COPD, plays a role in the pathological changes that cause damage to lung tissue compartments and structures.[3]

The global prevalence of COPD is estimated to approach 600 million cases worldwide by 2050, representing a relative growth of 23% compared to 2020. The greatest growth is occurring among women and in low- and middle-income countries.[4] In 2020, COPD was the third most common causes of death.[2] Indonesia does not have data on the prevalence of COPD, but with the number of vehicle users and smokers rising yearly, the country is estimated to have a fairly high prevalence of COPD.[5]

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The risk factors for COPD are environmental conditions, genes, and economic status.[3] These risk factors can change based on location, differences in air pollution from each region, work environment, smoking habits, and the introduction of new forms of smoking, such as vaping.[6]

Many studies have shown a relationship between exposure to motor vehicle gas emissions and human health. Several combustion products such as Carbon Monoxide (CO), Carbon (C), Lead (Pb), Aldehydes (RCHO), and Benzo(a)pyrene cause respiratory diseases, such as COPD.[7,8] Increased respiratory rate (RR), chest tightness, coughing, sputum, and a decline in Pulmonary Function Test (PFT) results are found in most people exposed to combustion gases.[9] Gas emissions are waste substances produced by hydrocarbon fuel from engines due to incomplete combustion. The growth of human civilization and the increasing variety and number of vehicles are some of the causes of the rising air pollution in cities, contributing to health problems.[8,10] A large number of vehicles have the potential to produce gas emissions due to the lack of vehicle maintenance, fuel type, and poor combustion.[8]

Oxidative stress caused by the imbalance between the production of reactive oxygen species (ROS) and the effective endogenous antioxidant mechanisms plays an important role in the initiation and development of various diseases. This imbalance disrupts cellular homeostasis thereby damaging important biological molecules such as DNA, proteins and lipids.[11] Oxidative stress due to chronic inflammation can be caused by two pathological processes: the respiratory tract becomes smaller, and the lung parenchyma is damaged, resulting in emphysema.[12] Pro-inflammatory cytokine IL (interleukin) 1α and tumour necrosis factor-alpha (TNF-α) contribute to COPD. Cytokines are not specific biomarkers for a disease, but cytokines are considered surrogate biomarkers for COPD because of their role in COPD related to the inflammatory response. IL-1 is the main cytokine produced by macrophages and respiratory epithelium, and its release, followed by IL-6, IL-8 and TNF-α, can cause neutrophilia, macrophage

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activation, and a response from T cells.[13] Pro IL-lP must go through a cleaving process and become biologically active by caspase-1 through activation of the nucleotide-binding domain (NOD)-like receptor protein (NLRP)3.[14] These binding sites are thought to be the binding sites for hypoxia-induced factor-1 (HIF-1) intended to provide the hypoxia-inducing upregulation of IL-lP.[15]

Hyperbaric Oxygen Therapy (HBOT) is a therapy that provides oxygen (O_2) at levels approaching '100%' in a high-pressure air chamber of more than 1 absolute atmosphere (ATA). HBOT is expected to increase pulse oxygen saturation (SpO_2) and the activity of the antioxidant enzyme Superoxide Dismutase (SOD) due to the production of free radicals or Reactive Oxygen Species (ROS) at a certain level and the use of ROS as a second messenger in the process. Therefore, HBOT can be a breakthrough therapy in treating breathing disorders and improving lung function damage.[16] The effect of HBOT on lung function has been investigated. HBOT increases PEF (maximum respiratory flow or peak expiratory flow) and FVC (Forced vital capacity).[17] This research examines strategies to improve lung tissue inflammation caused by oxidative stress in animal models with COPD due to motor vehicle fume exposure.

Materials and Methods

Study design

The study employed a "randomised control post-test design". Female Wistar rats (*Rattus novergicus*) between 6 and 8 weeks old, weighing 180-200 g and meeting the predetermined inclusion and exclusion criteria were used for the study. Thus, the experimental animals were close to homogeneity.[9] The study's sample size (n) was obtained based on preliminary research results. As many as 21 Wistar rats were divided into 3 groups: the negative control group (G0), the positive control group (G1), and the treatment group (G2). The G0 group was not given the HBOT intervention and was not exposed to motor vehicle gas emissions. Group G1 was not given the HBOT intervention but was exposed to motor vehicle gas emissions. Group G2 was exposed to motor vehicle gas emissions and given the HBOT intervention. In these three groups, blood serum samples were taken to analyze SOD activity and IL-1β levels, while, the lungs alveoli tissues were taken to analyze changes in the number of inflammatory cells in the lung alveoli tissue.

Ethical approval

The ethical approval for this study was obtained from the Research Ethics Commission of the Naval Health Institute Drs. R. Rijadi S., Phys, Surabaya, Number 01/EC/LKS/I/2024. The research was conducted at the Hyperbaric Animal Research Laboratory of the Naval Health Institute Drs. R. Rijadi S., Phys and Biochemistry Laboratory, Faculty of Medicine, Hang Tuah University, Surabaya between February and June 2024.

Animal models of COPD

The COPD animal models followed the procedures from He *et al*.[9] A total of 21 Wistar rats were acclimatized to the laboratory condition for 14 days. The G1 group was exposed to motor vehicle fumes, and the G2 group was exposed to vehicle gas (smoke) emissions and then treated with HBOT. The rats were exposed to smoke from a Honda PM 2.5 motor vehicle 5 days a week for 30 days. The rats were exposed to motor vehicle emissions for 1 h, followed by 30 min of rest, during 08.00-09.00, 10.00-11.00, 14.00-15.00, and 16.00-17.00 Western Indonesian Time.[9]

HBOT procedure

On the 45th day, the COPD animal models from the G2 group were placed in an animal chamber specifically made for experimental animals. The high-pressure air chamber used during HBOT was made of steel, and the conditions set inside the chamber were a room temperature of 27°C and an air humidity of 0.5. In this case, the animals inhaled 98.2% O_2 1.3 ATA 3 times at 30-minute, intervals 2 times at 5-minute breathing normal air, for 5 consecutive days, with 2 days of rest and then 5 more consecutive days. Overall, the subjects received a total of 10 days of treatment, thereafter, the animals (group G2) were returned to their original cages after each HBOT treatment.

Sample collection

On day 47, 30 minutes after the G2 treatment group completed the HBOT, all experimental animals were anaesthetized by an intraperitoneal injection of 50-100 mg of ketamine and 10 mg of xylazine per kilogram of body weight. After the rats were anaesthetized for 10 minutes and did not show a pain response, their blood was drawn using a syringe through the heart ventricle slowly to prevent collapse of the heart using a cardiac puncture technique with a syringe and a 23 G needle.[18] After that, a sample of their lung tissue was taken for histopathological examination.

Superoxide dismutase examination

The rats' SOD activity levels in the serum were estimated using the enzyme-linked immunosorbent assay (ELISA) method. To produce superoxide radicals, a reaction between Xanthine and Xanthine oxidase is required. In this assay, Nitroblue tetrazolium is reduced by superoxide radicals to formazan (purple). SOD could inhibit the reduction of Nitroblue tetrazolium through the superoxide radical reaction to produce O_2 and H_2O. Measurements were carried out using a BioVission Assay Kit Superoxide Dismutation spectrophotometer using the colourimetric method.[19]

Interleukine-1β examination

Measurement of levels or concentrations of IL-1β with ELISA Test. Blood samples were inserted into a plain venoject size 5 mL and centrifuged at 3000 rpm for 15 minutes to separate serum from sediment/pellets within 30 minutes after collection. The serum obtained was stored at a temperature of -20°C, if not analyzed immediately. Thereafter, an ELISA test was carried out to determine IL-1β levels using Reagen ELISA IL-1β (BT LAB) and quantification using an ELISA Reader (iMark Microplate Absorbance Reader).[19]

Histopathological examination

The euthanized rats were attached to a dissection board with lab tape. A vertical incision was made through the diaphragm and the ribs cut through to the chest cavity, exposing the heart and lungs. This was followed by a small incision made in the left chamber of the heart. Histopathological examinations of the rats' lung tissue were conducted using the hematoxylin-eosin (HE) staining technique. Organs that have been collected were fixed in a 10% formalin solution. The fixed samples were dehydrated in graded ethanol concentrations of 70%, 80%, 96%. The samples were washed in xylol and embedded in paraffin wax, then sliced using a microtome with a thickness of 5 µm. The slices were placed in a tissue bath, then taken with a glass object and then incubated in a slide warmer. Histological sections were stained with hematoxylin eosin (HE). After washing, the sections were stained with an eosin background and covered with a coverslip. Observations were carried out with an Olympus® light microscope with a x400 magnification and followed by taking photographs.[20]

Statistical analysis

Data were analyzed statistically using IBM SPSS version 22.0. p-value < 0.05 was considered significant.

Results and Discussion

This study used a low pressure of 1.3 ATA for the HBOT because the lung tissues in COPD patients are inflamed. Therefore, a higher pressure could risk pulmonary barotrauma. According to Singer *et al*., a high partial pressure of O_2 (PO_2) gives rise to reactive oxygen species (ROS).[21]Moreover, HBOT at higher pressures can pose a risk of hypoventilation and hypercarbia, as reported by Gawdi & Cooper.[22] High PO_2 can also cause hypoventilation and increased ventilation/perfusion (V/Q) mismatch. COPD is still considered a relative contraindication to HBOT. Thus, the risks versus benefits for COPD patients undergoing HBOT must be managed appropriately.[22]

This research revealed that the mean level of IL-1β in the COPD group given HBOT decreased, although not significantly compared to the

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COPD group not given HBOT. The mean level of SOD activity in the COPD group given HBOT increased significantly compared to the COPD group not given HBOT. Meanwhile, the histopathology results showed that the mean expression of inflammatory cells in the pulmonary alveoli tissue of the COPD group given HBOT decreased significantly compared to the COPD group not given HBOT. Macrophages can change their phenotype, similar to M1 and M2 macrophages, thereby influencing their function by several factors that play a role in the inflammatory response. M1 plays a role mainly in pro-inflammatory responses, and M2 macrophages in anti-inflammatory responses.[23]

The study revealed a significant difference in the mean levels of IL-1β in the different experimental groups, with a p-value of 0.048 ($p< 0.05$) in the G2 compared to G0. The Games-Howell test also showed that the mean IL-1β levels in G1 increased significantly with a p-value of 0.001 ($p > 0.5$) compared to G0. Additionally, the mean IL-1β level in G2 did not significantly decrease with a p-value of 0.544 ($p > 0.05$) compared to G1 and increased significantly compared to G0 at a p-value of 0.003 ($p< 0.05$). The differences in mean, SD, and ANOVA test results in IL-1β levels for groups G0, G1, and G2 are shown in Table 1.

The reactive oxygen species, superoxide, produced by HBOT by membrane-bound NADPH oxidase or mitochondrial electron transfer chain (ETC) were converted into H_2O_2 by the antioxidant SOD, which inhibits M1 macrophage polarisation and activates M2 macrophage polarisation via the signal transducer and activator of transcription 6 (STAT6) with a cysteine residue (Cys528) as a redox switch. These processes result in a decrease of the pro-inflammatory cytokine IL-1β. In addition, Cu, and Zn-SOD mediate the polarisation from M1 macrophages to M2 macrophages, which can be changed by modulating H_2O_2 generation. Differentiation of L arginine metabolism is characteristic of M1 and M2. The expression of Cu, Zn-SOD leads to the decreased gene expression of inducible nitric oxide synthase (iNOS) and NO synthesis while enhancing the production of arginase-1 and urea.[24] Furthermore, statistical analysis showed a significant difference between the mean SOD levels in the different study groups. G1 decreased significantly compared to G0 with a p-value of 0.037 ($p < 0.05$). Meanwhile, the Games-Howell test showed that the mean SOD levels in G1 decreased significantly compared to G0 with a p-value of 0.000 ($p < 0.5$). The mean SOD levels in G2 increased significantly compared to G1 at a p-value of 0.001 ($p < 0.05$) and increased significantly compared to G0 at a p-value of 0.001 ($p< 0.05$). The differences in mean, SD, and ANOVA analysis results in the SOD levels for groups G0, G1, and G2 are shown in Table 1.

According to He (2015) and Harnanik *et al*.[25] hyperbaric oxygen can stimulate excessive M1eff (CD80) to switch phenotype to M2reg (CD163). The repeated activation of either M1 or M2 impacts the host, and understanding how to suppress M1-M2 in disease regulation is very useful.[24,26] The ROS produced by HBOT reduces oxidative stress by stimulating increased SOD antioxidant activity and decreasing the inflammatory factor IL-1β.[27-28] HBOT significantly reduces the inflammatory cytokine IL-1β through several transcription factors, including the hypoxia Inducible Factor I (HlF-1) and nuclear factor kappa-light-chain-enhancer (NFkB) of B cells.[29] Similarly, a reduction in inflammation can reduce tissue damage and lung function and statistical analysis of the results obtained for the expression of inflammatory cells in the G0, G1, and G2 groups are shown in Table 1 and Figure 1. There was a significant difference in the expression of inflammatory cells in the lung tissues of rats in the G0 group compared to the G2 group with a p-value of 0.023 ($p< 0.05$). Meanwhile, the Games-Howell test showed that the mean expression of inflammatory cells in G1 increased significantly compared to G0 with a p-value of 0.013 ($p < 0.05$). Whereas, there was a decrease in the mean expression of inflammatory cells in G2 compared to G1 with a p-value of 0.029 ($p< 0.05$) and did not increase significantly compared to G0 at a p-value of 0.554 ($p > 0.05$).

Table 1: Differences in IL-1β levels, SOD activity and inflammatory cell expression in the research groups

Parameters	Group	Mean ± SD	MANOVA	Games-Howell		
				G0	G1	G2
IL-1β (ng/L)	G0	13.286 ± 4.461	*0.048		*0.001	*0.003
	G1	51.714 ± 15.147		*0.001		0.544
	G2	43.143 ± 14.565		*0.003	*0.544	
SOD (µl/ml)	G0	69.560 ± 3.514	*0.037		*0.000	*0.000
	G1	8.714 ± 2.430		*0.000		*0.001
	G2	32.714 ± 3.537		*0.000	*0.001	
Cell Inflammation	G0	177.286 ± 16.550	*0.023		*0.013	0.544
	G1	265.286 ± 56.423		*0.013		*0.029
	G2	190.429 ± 27.428		0.544	*0.029	

Notes * Indicates a significant difference (p < 0.05); SD : Standard Deviation

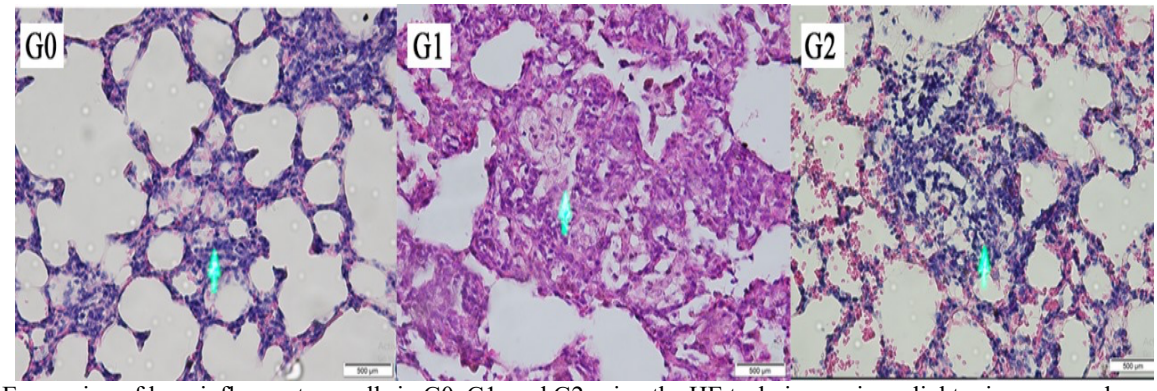

Figure 1: Expression of lung inflammatory cells in G0, G1, and G2 using the HE technique using a light microscope, shown at 500 µm magnification. Inflammatory cells appear swollen with enlarged cytoplasmic volume

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Conclusion

The results of this study revealed that HBOT has the potential to reduce oxidative stress caused by inflammation and hypoxia, which leads to lung tissue damage repairs caused by environmental toxicants (car emissions, CO, etc). As such HBOT could be an option in the management of COPD patients and can significantly improve their clinical symptoms. Further studies may aim at using HBOT together with existing standard therapies to prevent and treat COPD in animals and human models utilising more biomarkers and various doses of HBOT to identify the optimal dose and maintenance of HBOT therapy for COPD.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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