RESEARCH ARTICLE

Intermittent Exposure to Hypobaric Hypoxia Increases VEGF, HIF-1α, and Nrf-2 Expressions in Brain Tissue

Wardaya[1,2,3], Dewi Sukmawati[4], Nurhadi Ibrahim[5], Frans Ferdinal[6], Ninik Mudjihartini[7,8], Mohamad Sadikin[7,8], Sri Widia A. Jusman[7,8], Irawan Satriotomo[9], Wawan Mulyawan[2,10,*]

[1]Doctoral Program in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No. 6, Jakarta 10430, Indonesia
[2]Indonesian Air Force Institute of Aviation Medicine (Lembaga Kesehatan Penerbangan dan Ruang Angkasa/LAKESPRA dr. Saryanto), Jl. MT Haryono No. 46, Jakarta 12770, Indonesia
[3]Faculty of Health Sciences Universitas Dirgantara Marsekal Suryadarma, Jl. Halim Perdana Kusuma No. 1, Jakarta 13610, Indonesia
[4]Department of Histology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No. 6, Jakarta 10430, Indonesia
[5]Department of Physiology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No. 6, Jakarta 10430, Indonesia
[6]Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Taruma Negara, Jl. Taman S. Parman No. 1, Jakarta 11440, Indonesia
[7]Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No. 6, Jakarta 10430, Indonesia
[8]Center of Hypoxia and Oxidative Stress Studies, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No. 6, Jakarta 10430, Indonesia
[9]Department of Neuroscience, University of Florida, Gainesfille, Florida (FL) 32611, United States of America
[10]Aviation Medicine Program, Department of Community Medicine, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No. 6, Jakarta 10430, Indonesia

*Corresponding author. Email: wawan.mulyawan24@ui.ac.id

Received date: Jan 8, 2025; Revised date: Mar 9, 2025; Accepted date: Mar 11, 2025

Abstract

BACKGROUND: Hypoxia inducible factor-1α (HIF-1α), nuclear factor erythroid 2-related factor 2 (Nrf-2), and vascular endothelial growth factor (VEGF), play a crucial role as neuroprotective factors. Currently, there is a lack of studies examining the biomolecular responses of the brain to intermittent hypoxia resulting from various pressures. This study was conducted to investigate the physiological responses, histopathological features, and cellular adaptive responses in the brains of rats that were intermittently exposed to hypobaric hypoxic conditions.

METHODS: Thirty male Sprague Dawley rats were divided into six groups: a control group and five treatment groups exposed to hypobaric hypoxia. The treatment groups were placed in a hypobaric chamber simulating an altitude of 3,048 meters for 1 hour/day for 1, 7, 14, 21, and 28 days. After exposure, brain tissue was collected for histopathological analysis and protein quantification of HIF-1α, Nrf-2, cytoglobin (Cygb), neuroglobin (Ngb), VEGF, malondialdehyde (MDA), glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT).

RESULTS: In the brain, intermittent hypobaric hypoxia significantly increased HIF-1α expression (p=0.000) and its downstream proteins Cygb (p=0.000), and VEGF (p=0.001), with a peak at 14x IHH exposure compared to control. This was followed by a significant increase in Nrf-2 expression (p=0.000), SOD (p=0.000), Gpx (p=0.000), and CAT activity (p=0.000), indicating an adaptive antioxidant response. Conversely, MDA levels was decreased with prolonged exposure, suggesting reduced oxidative damage.

CONCLUSION: IHH elevates HIF-1α, Nrf-2, and oxidative stress markers, triggering an adaptive antioxidant response in the rat's brains.

KEYWORDS: HIF-1α, intermittent hypobaric hypoxia, Nrf-2, oxidative stress

Indones Biomed J. 2025; 17(2): 197-206

Introduction

Hypobaric hypoxia can occur due to decreased atmospheric pressure. Those conditions can be experienced by everyone who is at high altitudes.(1) Pilots who use unpressurized cabin aircraft (helicopters and Cessna) are vulnerable to hypobaric hypoxic conditions. The effects of hypoxia will be visible in healthy people after reaching an altitude of 3,048 meters. Pilots who usually fly at low altitudes below 3,048 meters in unpressurized cabins will experience difficulty if they are suddenly required to fly over mountainous areas above 3,048 meters.(2)

The frequent exposure of pilots to hypobaric hypoxia (pressure of 523 mmHg) causes the risk of physiological changes in their bodies. A decrease in oxygen supply in the body can affect the condition of various organs that require an adequate supply of oxygen, such as the brain. The human brain utilizes about 20% of the body's oxygen supply. Reduced oxygen supply to the brain can cause decreased control, increased heart rate and blood pressure, changes in cognitive function, reduced short-term memory ability, and even fatal consequences, like coma and the damage to the structure and function of the brain.(3,4)

Hypoxic conditions can disrupt the electron transport in the mitochondria, which causes mitochondrial membrane leakage and produces reactive oxygen species (ROS). Overproduction of ROS can cause oxidative stress that triggers the activation of nuclear factor-erythroid-2 related factor 2 (Nrf-2). Nrf-2 is a transcription factor for antioxidant enzyme synthesis to counteract the increased formation of ROS.(5) It is known that hypoxia can activate the necrosis and apoptosis processes through ROS production. Increased ROS after hypoxia can damage DNA, cell membranes (lipid peroxidation), and proteins that cause the necrosis of brain cells.(6,7)

However, the body has compensatory mechanisms to overcome hypoxic conditions. One of those mechanisms is via hypoxia inducible factor-1α (HIF-1α) activation.(8,9) HIF-1α activation can regulate the expression of a number of genes to increase oxygen supply through erythropoiesis, glycolytic enzymes also glucose transporters.(10) HIF-1α also regulates the angiogenesis process by increasing the expression of the vascular endothelial growth factor (VEGF), myoglobin (Mgb) and cytoglobin (Cygb) genes, as well as neuroglobin (Ngb) to increase blood and oxygen flow as an adaptation to hypoxia.(11-13)

Various studies show cognitive impairment, difficulty concentrating, impaired memory processes, and decreased vision in pilots exposed to hypoxic conditions.(14-16) However, these various studies have not been supported with molecular processes explanation that occur in body tissues, especially the brain, when exposed to continuous hypoxic conditions. This study was conducted to examine the physiological responses, histopathological features, and cellular adaptive responses in the brain of rats exposed to hypobaric hypoxic conditions intermittently (3,048 meters; 523 mmHg) for 1 hour/day for 7, 14, 21, and 28 days compared to transient hypobaric hypoxic conditions.

Methods

Study Design

An experimental *in vivo* investigation study utilizing thirty male Sprague-Dawley rats aged between 2 to 3 months, with body weights ranging from 200 to 250 grams, was conducted. The rats were randomly divided into six groups, comprising of a control group and five treatment groups. The control group was housed in a standard environment, while the treatment groups were exposed to hypobaric hypoxia conditions at an altitude equivalent to 3,048 meters (523 mmHg pressure). The acute hypobaric hypoxia (AHH) group was subjected to a single exposure for one hour in one day. The intermittent hypobaric hypoxia (IHH) groups underwent repeated daily exposures of 1 hour/day for either 7 days (IHH-7x), 14 days (IHH-14x), 21 days (IHH-21x), or 28 days (IHH-28x).

Fifty mg/kg ketamine and 5 mg/kg xylazine were utilized for euthanasia, after which the brains were extracted and stored at -20°C. All procedures were conducted at the Indonesian Air Force Institute of Aviation Medicine (*Lembaga Kesehatan Penerbangan dan Ruang Angkasa*/ LAKESPRA dr. Saryanto), the Department of Biochemistry and Molecular Biology, and the Department of Histology, Faculty of Medicine, Universitas Indonesia. Approval for the study was obtained from the Research Committee of the Faculty of Medicine, Universitas Indonesia (No. KET-943/ UN2.F1/ETIL/PPM.00.02/2021).

Cognitive Function Measurement

After completing the hypobaric hypoxic treatment, the rat underwent cognitive function tests with the Y Maze method. The rats were trained to determine the aisle containing food. This test aimed to assess spatial working memory. The alley's walls were coloured black and white. The black alley had a food at the end of the hallway. Rats should had learnt to go to the location with food depending on the colour of

the walls. One day before the IHH treatment, rats were tested 3-times with 5-minute intervals, the test duration after IHH treatment was measured and compared with the control.

Histology and Immunohistochemistry (IHC)

Immediately following surgical resection, a part of brain tissues were preserved with 10% neutral buffered formalin (NBF). Subsequently, the brain tissue was embedded in paraffin and then cut into 4-5 μm thick sections using the microtome. The slides were dewaxed, rehydrated, and rinsed in PBS twice for 5 minutes. The haematoxylin-eosin (HE) staining and IHC were applied to the brain tissue slides. Heat-based antigen retrieval in Tris-EDTA buffer, pH=9, was carried out for 20 minutes in a decloaking chamber (96°C) as a pre-treatment for IHC. After that the slides were soaking in 3% hydrogen peroxide for 10 minutes, then submerged in blocking solution for a further 10 minutes. The sections were further incubated for 1 hour at 4°C with anti-VEGF (dilution 1:200, C-1; Cat. No. SC-7269; Santa Cruz Biotechnology, Dallas TX, USA) antibody. The secondary antibody reaction was performed for 30 minutes at room temperature with anti-rabbit antibody (Cat. No. RE7112; Leica Biosystem, Vista, CA, USA). The 3,3'-Diaminobenzidine (DAB) was used as a substrate to visualize positive signals. Positive control for VEGF was human tonsil tissue.

Evaluations of Histopathological Results

To analyse the effect of hypobaric hypoxia on rat brain tissue, the number of neuron from HE staining slides was measured. Observations were made in five small fields of view of each sample using light microscope at 400x magnification. As for the expression of VEGF, we measured the results of percentage of neuron that produce brown colour on brain cell membranes in five small visual fields. The IHC staining results were observed using an Olympus inverted IX73 microscope (Olympus, Tokyo, Japan). The H&E and IHC staining were documented using Optilab viewer 3.0 (PT Miconos, Sleman, Indonesia) and further analysed using Image Raster (PT Miconos), and Image J (National Institute of Health, Bethesda MD, USA), respectively.

Protein Isolation

The process of protein isolation commences with the preparation of tissue homogenate. Approximately 100 mg of brain tissue was homogenized in a 1X PBS (pH 7.4) solution using a homogenizer. The mixture was centrifuged at 5000×G for 5 minutes following homogenization. The resulting supernatant was carefully transferred to a new tube and utilized for the analysis of various research parameters including malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione (GSH), HIF-1α, Nrf-2, Cyg, Ngb. The concentration of total protein was determined by spectrophotometry at 280 nm.

Measurement of Oxidative Stress and Antioxidants Markers

MDA as an oxidative stress marker was measured using the Wills method, based on the reaction of thiobarbituric acid reactive substance (TBARS).(17) As for the antioxidants marker, in this study, levels of GSH, the activities of SOD, GPx, and CAT, were measured. The concentration of GSH was measured using the colorimetric method based on the reaction between 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and GSH. This method measured the levels of 5-thionitrobenzoic acid (TNB) resulting from that reaction, which was read at 412 nm wavelength.(18) Commercial kits RANSOD (Cat No. SD126; Randox Laboratories, Crumlin, UK) and RANSEL (Cat No. RS505, Randox Laboratories) were used to measure the activities of SOD and GPx, respectively.(17,19) CAT activity was measured based on the reaction with hydrogen peroxidase (H_2O_2). The H_2O_2 was then converted into oxygen and hydrogen by the catalase in the sample. The decreasing levels of H_2O_2 was read using spectrophotometer at 240 nm.(17)

The Measurement of HIF-1α, Nrf-2, Cygb, and Ngb Expressions

The expression levels HIF-1α, Nrf-2, Cygb, and Ngb expression were measured using following enzyme-linked immunosorbent assay (ELISA) kits: HIF-1α (Cat No. E-EL-RO513; Elabscience, Houston, TX, USA), Nrf-2 (Cat No. E-EL-R1052; Elabscience), Cygb (Cat No. MBS2890631; MyBiosource, San Diego, CA, USA), and Ngb (Cat No. ER1190; FineTest, Wuhan, China) with sandwich ELISA method. The assay followed the manufacturer's instructions, where target proteins in brain tissue homogenates bound to pre-coated capture antibodies, followed by detection with biotinylated antibodies and a streptavidin-HRP system. Absorbance was measured using a microplate reader at 450 nm. Concentrations of HIF-1α, Nrf-2, Cygb, and Ngb were quantified using the AATbio ELISA calculator (AAT Bioquest, Pleasanton, CA, USA), employing a four-parameter logistic regression analysis. To ensure accuracy, all data were normalized based on total protein concentration.

Results

Effect of Hypobaric Hypoxia on Cognotive Function

The results of this study showed that after hypobaric hypoxia treatment for AHH and IHH-7x, there was an increase in the length of time rat walks to find the right aisle containing food. The longest rats were able to find food were in the group IHH 14x. However, after exposure IHH 21x and 28x, there was an improvement in the rat walking time scoring in their ability to find the right aisle containing food. From the statistical test results, there were no significant differences (p=0.057) in the cognitive function abilities of rat after IHH treatment (Figure 1).

Histopathological and VEGF Expression Changes in Brain Cells Following Hypobaric Hypoxia Treatment

The results of HE staining showed differences in the number of cells in the brain after hypobaric hypoxia treatment compared to the control group (Figure 2). The results showed that the number of cells in the brain after hypobaric hypoxia treatment significantly differed between the control group and the group AHH (p=0.010), IHH 7x (p=0.012), IHH 14x (p=0.008), IHH 21x (p=0.030), and IHH 28x (p=0.009). However, the differences between treatment groups were different but not significant. The results of the current study showed significantly different VEGF expression in brain cells after hypobaric hypoxia treatments (AHH, IHH 7x, IHH 14x, IHH 21x, and IHH 28x) with control. The VEGF expression of the AHH treatment group was significantly higher than the group IHH 7x. The result also showed that the VEGF expression of IHH 7x times groups was significantly lower than IHH 21x (p=0.010) and IHH 28x (p=0.000) groups. However, the VEGF expression of the AHH treatment groups with IHH 14x (p=0.320), IHH 21x (p=0.970), and IHH 28x (p=0.076) were not statistically significant (Figure 3).

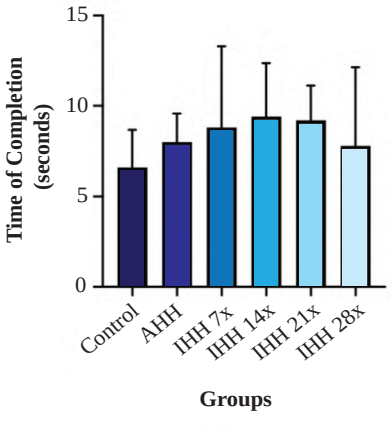

Figure 1. Time of completion for cognitive test in control and treatment groups. Tested with ANOVA, followed by Tukey's test. n=5 in each group.

Hypobaric Hypoxia Increased HIF-1α, Nrf-2, Cygb, and Ngb Expressions in The Brain

Figure 4 represented the results of HIF-1α, Nrf-2, Cygb, and Ngb level measurements. The results of this study showed that the brains of rats treated with hypobaric hypoxia had higher levels of HIF-1α than the control group. The results showed that the highest levels of HIF-1α were found in the IHH 14x group. A similar pattern was also found in Nrf-2 levels. Nrf-2 levels in all treatment groups tended to be higher than the control except in 28x IHH group. The highest Nrf2 level was also found in the group with IHH 14x treatment. The results also showed that the Cygb levels in the treatment group were significantly higher than the controls. Cygb levels tended to show a pattern that was in line with HIF-1α and Nrf-2 levels, which increased in AHH and reached its peak at IHH 14x, then decreased at IHH 21x and 28x. Meanwhile, Ngb levels tended to show a different pattern. The results showed that the Ngb level in the AHH group was significantly lower than the control, but then it was significantly higher in the IHH 7x, 14x, 21x, and 28x groups than in control.

Increased Antioxidant Enzyme Activities and GSH Levels in Brain Following Hypobaric Hypoxia Exposure

The activity of SOD, CAT, and GPx as endogenous enzymatic antioxidant markers were measured and shown in Figure 5A-5C. The results showed higher SOD activity in the rat brain of the treatment group under hypobaric hypoxic conditions compared to the control group. The 14x IHH group showed the most high SOD activity. A similar pattern was found in the GPx enzyme activity. The results showed that the GPx activity tends to be higher in all treatment groups than in control. The most high GPx activity was also found in the IHH 14x group. Different results showed in CAT activity. The CAT enzyme in the treatment group tended to have lower activity than in control group. The lowest CAT enzyme activity was found in the AHH group.

In this study, the GSH level was also measured, and it found that GSH levels in the treatment group under hypobaric hypoxic conditions were higher than the control group. The highest level was in the group (IHH 14x). The results showed that GSH level of AHH, IHH 7x, IHH 21x and IHH 28x groups were significantly lower than IHH 14x group (Figure 5D).

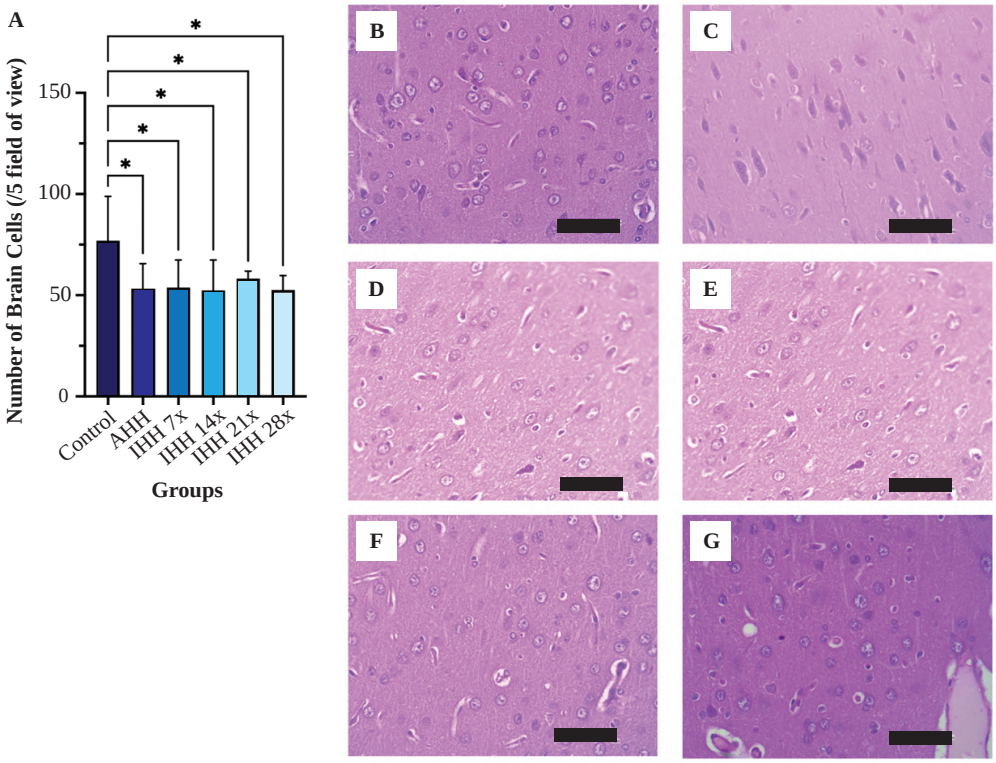

Figure 2. Number of brain cells in control and treatment groups. A: Number of rat brain cells per 5 fields of view. Tested with ANOVA, followed by Tukey's test. *Significant if $p<0.05$. n= 5 in each group. B-G: HE staining of: control group (B), AHH group (C), IHH 7x group (D), IHH 14x group (E), IHH 21x group (F), IHH 28x group (G). Black bar: 5 µm.

Increased MDA Levels in Rat Brain Following IHH Exposure

Figure 6 showed MDA levels in the brains of rats exposed with hypobaric hypoxia. The results showed higher levels of MDA in the brains of rats exposed to hypobaric hypoxia compared to the control group. The highest MDA levels were shown in IHH 28x group. The MDA level in rat brains exposed to AHH, IHH 21x and IHH 28x were significantly higher compared to the control group. The MDA level of IHH 7x group was significantly lower compared to the IHH 14x and 28x groups.

Correlation Analysis of HIF-1α with Cygb, Ngb, Nrf-2, Antioxidants, MDA, and VEGF in Rat Brain

The correlation between certain parameters in this study was presented in Table 1. The findings revealed a moderate and statistically significant positive correlation between the levels of HIF-1α and Nrf-2 in the rat brains (r=0.599, p=0.000). Similarly, a moderate and positive correlation was observed between the levels of HIF-1α and VEGF in brain tissue (r=0.401 p=0.027). Additionally, the results demonstrated a moderately significant negative correlation between GSH levels and brain MDA levels (r=-0.407, p=0.025).

Discussion

Cognitive decline in this study was reflected by increased completion time in the Y-maze test. Although not statistically significant, AHH, IHH 7x, and IHH 14x extended the completion time. Improvement began at IHH-21 and peaked at IHH 28x. The result of this study showed that cognitive function did not experience significant changes after treatment. However, individually, several rats showed an extension of reaction time of up to 3 seconds. Different results were previously found in a study where IHH only performed as much as 4 times.(20) Other study even found better results in the IHH group than in normoxia experimental animals.(21) In the current study, there was a slowdown in cognitive function, which then improved at IHH 28 times (Figure 1). This decrease in cognitive function can be expected to be influenced by the decrease in oxygen saturation in hypoxic conditions. Brain histology analysis in this study was carried out in the frontal area.

The results of HE staining showed a significant decrease in the number of brain cells per field of view in the group exposed to hypobaric hypoxia (AHH; IHH 7x; IHH 14x; IHH 21x; and IHH 28x). However, VEGF expression

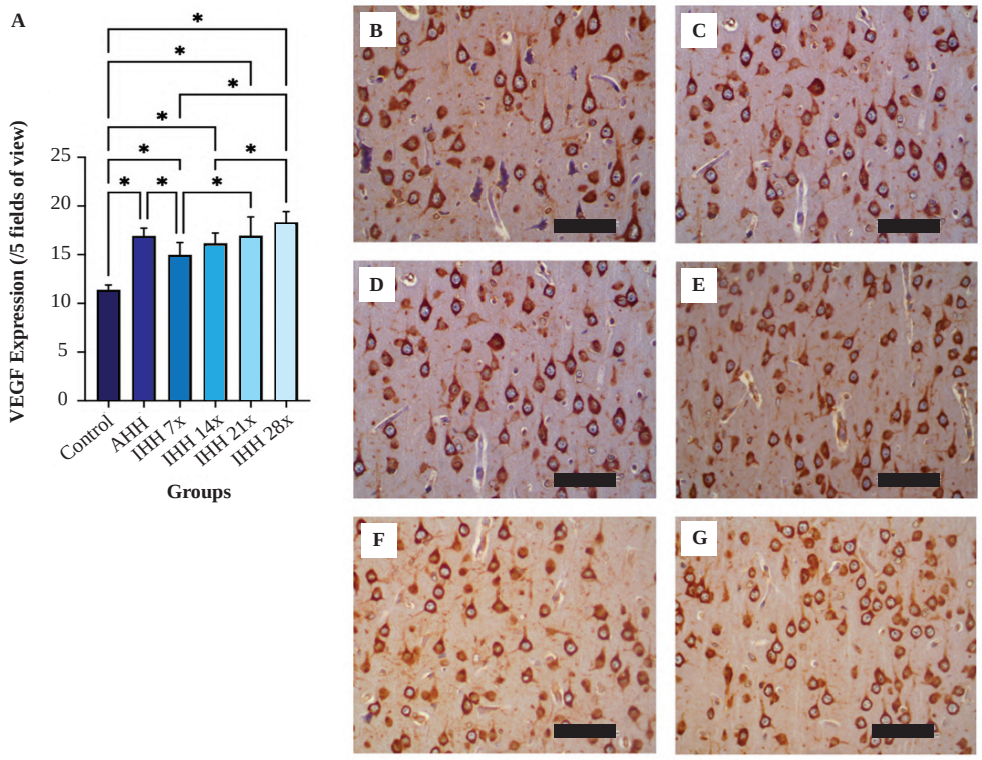

Figure 3. Number of neurons that express VEGF in control and treatment groups. A: Percent VEGF expression from five fields of view of rat brain. Tested with ANOVA, followed by Tukey's test. *Significant if $p<0.05$. n= 5 in each group. B-G: VEGF expression from: control group (B), AHH group (C), IHH 7x group (D), IHH 14x group (E), IHH 21x group (F), IHH 28x group (G). Black bar: 5 µm.

significantly increased in the AHH group, declined in the IHH 7x group, and then progressively rose with higher IHH (7x, 14x, and 21x) exposure, peaking at IHH 28x. This can be caused by hypobaric hypoxia, which can reduce oxygen intake so that oxygen reaching the tissues is reduced. However, organisms, including the brain, are equipped with mechanisms to overcome this. The significant and persistent increase over 28 days of HIF-1α suggests this.

Hypoxia in the brain affects the function of this organ, which is more or less reflected in a decrease in cognitive

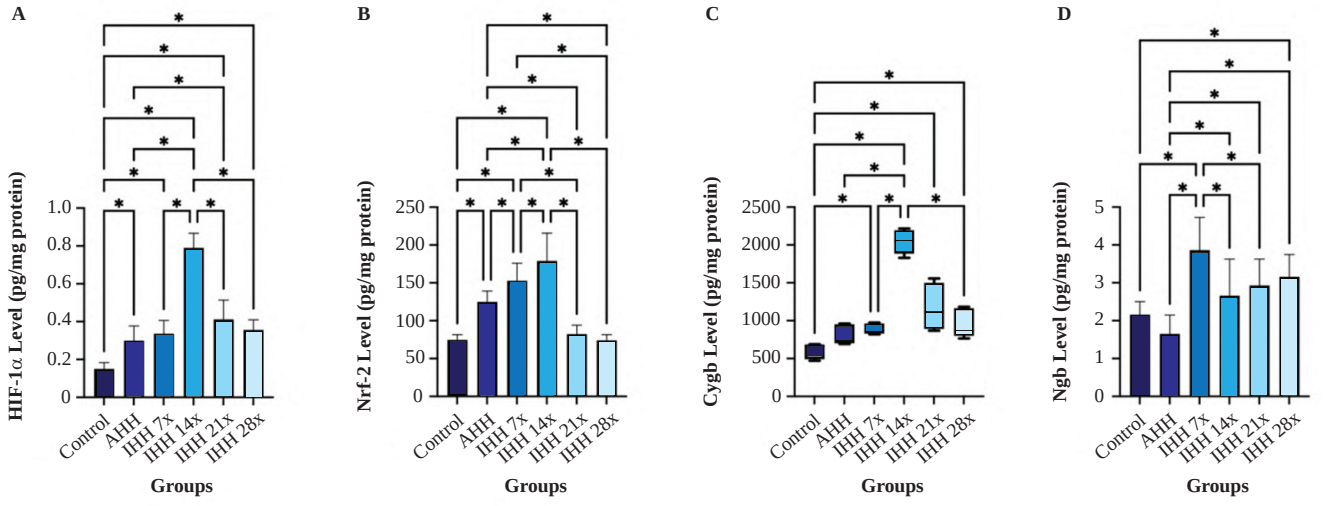

Figure 4. The expression of HIF-1α, Nrf-2, Cygb, and Ngb in rat brain exposed to hypobaric hypoxia for one hour 1, 7, 14, 21, 28 days. A: HIF-1α expression. B: Nrf-2 expression, C: Cygb expression. D: Ngb expression. Tested with ANOVA, followed by Tukey's test (for Figures A, B, and D); Tested with Kruskall-Wallis, followed by Mann-Whitney test (for Figure C). *Significant if $p<0.05$. n= 5 in each group.

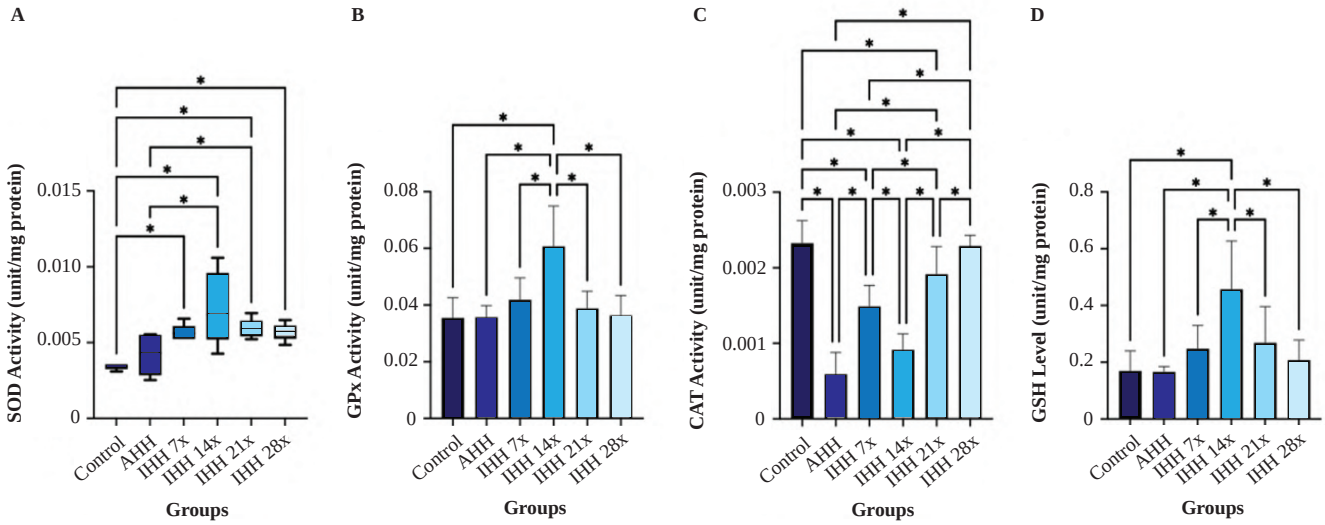

Figure 5. The effect of hypobaric hypoxia exposure to endogenous antioxidants in rat brain. A: The activity of SOD. B: The activity GPx. C: The activity CAT. D: the level of GSH. Tested with Kruskall-Wallis, followed by Mann-Whitney test (for Figure A). Tested with ANOVA, followed by Tukey's test (for Figures B, C, and D). *Significant if $p<0.05$. n= 5 in each group.

function, although it is not significant. Under intermittent hypoxia due to hypobaric conditions, oxygen deprivation stabilizes HIF-1α by inhibiting its degradation, allowing it to dimerize with HIF-1β and activate VEGF transcription, promoting angiogenesis to improve oxygen supply. Simultaneously, hypoxia-induced oxidative stress releases Nrf-2 from its inhibitor Keap1, enabling it to upregulate antioxidant enzymes, such as SOD, for cellular protection. The combined effects of VEGF-mediated vascular adaptation and Nrf-2-driven oxidative defense enhance brain resilience to hypoxic stress.

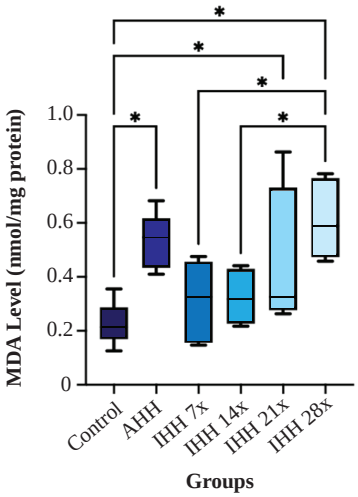

Figure 6. The effect of hypobaric hypoxia exposure to MDA level in rat brain. Tested with Kruskal Walis, followed by Mann-Whitney test. *Significant if $p<0.05$. n= 5 in each group.

In this study, HIF-1 expression significantly increased in all hypobaric hypoxia groups (either AHH or IHH groups) compared to the control, peaking in the IHH 14x group. Another study also proved that HIF-1α increased in mice induced by hypobaric hypoxia equivalent to an altitude of 11,000 meters. HIF-1α activation can increase cerebral microvascular density in overcoming hypoxic conditions in brain cells.(22) Exposure to hypobaric hypoxia equivalent to an altitude of 8,000 meters occurs as a process of adaptation of glutamate receptors without change in cognitive function between the treatment and control groups.(23) HIF-1α, which is a transcription factor that controls several proteins to overcome hypoxia, such as Ngb, Cygb, and VEGF (24), succeeded in increasing the expression of these proteins and maintaining it for 28 days.

Ngb is a type of extra-erythrocyte hemoglobin which is mainly found in the central nervous system. Ngb shows the characteristics of a specific O_2 transport protein, its spectrum image changes when the Fe^{2+} in its Hem core undergoes oxidation and reduction and also when the Hem core binds CO or CN^-.(25,26) In this study, changes in Ngb levels occurred in the form of increases and decreases. Ngb levels declined in the AHH group, then peaked at IHH 7x before gradually decreasing from IHH 14x to IHH 28x, approaching normal group levels. It is very likely that Ngb also plays a role as an antioxidant in brain cells. The increase in Cygb in this study was due to a decrease in Ngb protein which occurred on the first day of hypobaric exposure (AHH) and continued until exposure to IHH 14 times. Referring to findings in previous research which

Table 1. Correlation analysis between HIF-1α, Cygb, Ngb, Nrf-2, SOD, GPx, CAT, GSH, MDA, and VEGF of rat brain exposed with hypobaric hypoxia.

Variables	*HIF-1α*	*Cygb*	*Ngb*	*Nrf2*	*SOD*	*GPx*	*CAT*	*GSH*	*MDA*	*VEGF*
HIF-1α										
Cygb	r=0.904									
	p =0.000*									
Ngb	r=0.135	r=0.154								
	p =0.478	*p* =0.415								
Nrf2	r=0.599	r=0.542	r=0.017							
	p =0.000*	*p* =0.001*	*p* =0.927							
SOD	r=0.724	r=0.589	r=0.133	r=0.466						
	p =0.000*	*p* =0.000*	*p* =0.482	*p* =0.009*						
GPx	r=0.703	r=0.616	r=-0.045	r=0.627	r=0.671					
	p =0.000*	*p* =0.000*	*p* =0.811	*p* =0.000*	*p* =0.000*					
CAT	r=-0.400	r=-0.420	r=0.232	r=-0.686	r=-0.087	r=-0.266				
	p =0.028*	*p* =0.021*	*p* =0.217	*p* =0.000*	*p* =0.648	*p* =0.154				
GSH	r=0.675	r=0.638	r=-0.082	r=0.540	r=0.679	r=0.800	r=-0.189			
	p =0.000*	*p* =0.000*	*p* =0.668	*p* =0.002*	*p* =0.000*	*p* =0.000*	*p* =0.318			
MDA	r=0.055	r=-0.025	r=0.048	r=-0.221	r=0.174	r=-0.175	r=-0.081	r=-0.408		
	p =0.773	*p* =0.896	*p* =0.801	*p* =0.241	*p* =0.356	*p* =0.354	*p* =0.670	*p* =0.025		
VEGF	r=0.401	r=0.326	r=0.101	r=0.028	r=0.391	r=0.010	r=-0.231	r=-0.025	r=0.721	
	p =0.027*	*p* =0.078	*p* =0.596	*p* =0.884	*p* =0.032*	*p* =0.957	*p* =0.219	*p* =0.895	*p* =0.000*	

The table displays correlation coefficients (r values) for each parameter. *Statistically significant correlations if $p<0.05$. Tested by Pearson Correlation analysis.

stated that Ngb and Cygb play a role in regulating oxygen supply to the brain, a decrease in Ngb levels followed by an increase in Cygb levels indicates the time when these two proteins play a role in hypoxic conditions.(27)

Most of the ROS in cells is formed in mitochondria during cellular respiration in the respiratory chain. ROS production is modulated by the increased flow of electrons through the respiratory complex. Apart from ROS, in hypoxic conditions nitric oxide (NO) is also produced which can produce other reactive nitrogen species (RNS). (28-30) The production of ROS/RNS in cells will cause the use of endogenous antioxidants, resulting in an imbalance of oxidants/antioxidants in acute hypoxia, IHH 21x and IHH 28x which is indicated by an increase in MDA and a decrease in GSH, causing oxidative stress. This is supported by the correlation results in Table 1 which show a moderate and significant negative relationship.

MDA is a product of cell membrane damage resulting from lipid peroxidation by free radicals which causes cell injury. GSH is a non-enzymatic endogenous antioxidant that works together with GPx to convert H_2O_2 into H_2O. If lipid peroxidation cannot be stopped by antioxidants, cell injury will continue to cell death. Hypoxia in cells or tissues causes an increase in oxidative stress. ROS or RNS produced by mitochondria in hypoxic conditions causes increased oxidative stress.(29) Several studies have proven that exposure to hypoxia increases lipid peroxidation by increasing MDA levels in the liver and kidneys of mice. (31,32)

On the other hand, MDA levels tend to increase, causing activation of Nrf-2, which activates enzymatic antioxidants such as SOD, GPx and CAT. Research conducted in continuous hypoxia showed that SOD in the rat brain increased with the duration of exposure.(33) Our prior study also demonstrated how the rise in MDA levels affects the activation of Nrf-2, leading to alterations in the activity of antioxidant enzymes in the hearts of rats subjected to IHH.(34) The results of current study showing significant increases in SOD and GPx in the rat brain exposed to hypobaric hypoxia compared to controls. Meanwhile CAT showed a decline. These results prove that CAT enzymatic activity plays a large role as the first line of defense in brain cells which reaches its lowest point at IHH 14x. Furthermore, together with GSH, SOD and GPx, it tries to

prevent lipid peroxidation, namely MDA, which continues to increase as the amount of IHH exposure increases.

Correlation test analysis of HIF-1α with Nrf-2 in brain cells (Table 1) shows a moderate and significant positive relationship. From these results, there is a directly proportional relationship between increasing HIF-1α protein levels in line with increasing Nrf-2 protein levels. As mentioned above, in brain cells, hypoxia induces oxidative stress and consequently reduces antioxidant levels because they are used to neutralize ROS. HIF-1α levels, Nrf-2 levels were highest in the IHH 14x group but then decreased to the lowest point at IHH 28x. The results of this current study were supported by another study that reported that Nrf2 activation in the IHH group was induced by oxidative stress, this transcription factor was activated in response to increased ROS. It can be said that 28 days of intermittent hypoxic conditions with a pressure of 523 mmHg equivalent to 3,048 meters for 1 hour/day can be overcome. by the formation of proteins that overcome hypoxia, namely Cygb, Ngb and VEGF as well as increased oxidative stress which can be overcome by enzymes coordinated by Nrf-2. This study had restricted number of mouse brain tissue samples, which limited the scope for measuring additional biochemical parameters. Further studies using the same animal model on synaptic plasticity in the hippocampus.

Conclusion

The results of this study indicate that intermittent exposure to air pressure of 523 mmHg does not impair cognitive function. However, it leads to changes in brain tissue histopathology, the expression of HIF-1α, Nrf-2, and their regulated proteins. Notably, the most significant alterations were observed after 14 days of exposure, marked by increased levels of HIF-1α, Cygb, and Ngb, followed by a rise in Nrf-2 and antioxidant enzymes. These findings provide valuable insights into brain's addaptive responses to hypobaric hypoxia, which is particularly relevant to pilots frequently exposed to such conditions.

Acknowledgments

We express our gratitude to the Chief of Staff of the Indonesian Air Force, the Indonesian Air Force Institute of Aviation Medicine (*Lembaga Kesehatan Penerbangan dan Ruang Angkasa*/LAKESPRA dr. Saryanto), and the entire team in the Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia. Additionally, we acknowledge the Direktorat Riset dan Pengabdian Masyarakat (DRPM) (No. BA-490/UN2.RST/PPM.00.03.01/2024) for the funding of this study.

Authors Contribution

MS, SWAJ, and WM conceptualized the study, supervised data collection and analysis, contributed to manuscript preparation and review. WW is responsible for data collection and analysis, as well as drafting, revising, and finalizing the manuscript. FF, IS, NI, NM, and DS provided valuable input during the manuscript drafting and review process. WM secured funding for the study. All authors contributed to the overall completion of the manuscript.

References

1. Aerospace Medical Association; Aviation Safety Committee; Civil Aviation Subcommittee. Cabin cruising altitudes for regular transport aircraft. Aviat Space Environ Med. 2008; 79(4): 433-9.
2. ICAO. Operation of Aircraft: Annex 6 to the Convention on International Civil Aviation. ICAO. 2005.
3. Semenza GL. Hypoxia-inducible factors in physiology and medicine. Cell. 2012; 148(3): 399-408.
4. Maiti P, Singh SB, Sharma AK, Muthuraju S, Banerjee PK, Ilavazhagan G. Hypobaric hypoxia induces oxidative stress in rat brain. Neurochem Int. 2006; 49(8): 709-16.
5. Cheng L, Zhang H, Wu F, Liu Z, Cheng Y, Wang C. Role of Nrf2 and its activators in cardiocerebral vascular disease. Oxid Med Cell Longev. 2020; 2020: 4683943. doi: 10.1155/2020/4683943.
6. Layal K. Peran Nrf2 dalam patogenesis stres oksidatif dan inflamasi pada penyakit ginjal kronik. Syifa'MEDIKA. 2016; 7(1): 16-24.
7. Mahmood M, Mushtaq M, Malik A, Khurshid R, Aamir F, Javed A. Oxidative stress mediates the association between thyroid dysfunction and breast cancer. Indones Biomed J. 2023; 15(4): 303-9.
8. Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC. Differential roles of hypoxia-inducible factor 1alpha (HIF-1α) and HIF-2α in hypoxic gene regulation. Mol Cell Biol. 2003; 23(24): 9361-74.
9. Widhiantara IG, Permatasari AAAP, Rosiana IW, Sutirtayasa IWP, Siswanto FM. Role of HIF-1, Siah-1 and SKN-1 in inducing adiposity for caenorhabditis elegans under hypoxic conditions. Indones Biomed J. 2020; 12(1): 51-6.
10. Chhabra V, Anand AS, Baidya AK, Malik SM, Kohli E, Reddy MPK. Hypobaric hypoxia induced renal damage is mediated by altering redox pathway. PLoS One. 2018; 13(7): e0195701. doi: 10.1371/journal.pone.0195701.
11. Krock BL, Skuli N, Simon MC. Hypoxia-induced angiogenesis: Good and evil. Genes Cancer. 2011; 2(12): 1117-33.
12. Kamga C, Krishnamurthy S, Shiva S. Myoglobin and mitochondria: A relationship bound by oxygen and nitric oxide. Nitric Oxide. 2012; 26(4): 251-8.

13. Siregar FM, Hardiany NS, Jusman SWA. Negative correlation between cytoglobin expression and intracellular ROS levels in human skin keloid fibroblasts. Indones Biomed J. 2019; 11(1): 48-51.
14. Pilmanis AA, Balldin UI, Fischer JR. Cognition effects of low-grade hypoxia. Aerospace medicine and human performance. 2016; 87(7): 596-603.
15. Heratika D, Kekalih A, Mulyawan W, Agustina A, Soemarko DS, Siagian M. The effect of the altitude zone on cognitive function for male pilots in indoctrination and aerophysiology training in 2019. Int J Appl Pharm. 2020: 12(Special 3): 12-4.
16. Bouak F, Vartanian O, Hofer K, Cheung B. Acute mild hypoxic hypoxia effects on cognitive and simulated aircraft pilot performance. Aerosp Med Hum Perform. 2018; 89(6): 526-35.
17. Dewi S, Sadikin M, Mulyawan W. Oxidative stress in the heart of rats exposed to acute intermittent hypobaric hypoxia. Ukr Biochem J. 2021; 93(3): 68-74.
18. Ekawati M, Mujihartini N, Jusuf AA, Dharmasetiawani N, Jusman SWA, Sadikin M. Altered expressions of endothelial junction protein of placental capillaries in premature infants with intraventricular hemorrhage. Med J Indones. 2016; 25(3): 143-50.
19. Ramadhani D, Suvifan VA, Purnami S, Rahajeng N, Lusiyanti Y, Wanandi SI, *et al*. Superoxide dismutase and glutathione peroxidase activities in a population exposed to high indoor radon concentration: a preliminary report. Free Radic Res. 2021; 55(11-12): 1094-103.
20. Tsai YW, Yang YR, Wang PS, Wang RY. Intermittent hypoxia after transient focal ischemia induces hippocampal neurogenesis and c-Fos expression and reverses spatial memory deficits in rats. PloS one. 2011; 6(8): e24001. doi: 10.1371/journal.pone.0024001.
21. Wittner M, Riha P. Transient hypobaric hypoxia improves spatial orientation in young rats. Physiol Res. 2005; 54(3): 335-40.
22. Mulyawan W. Analisis respons adaptasi jaringan otak pasca induksi hipoksia hipobarik intermitten pada tikus: kajian khusus terhadap ekspresi hypoxia inducible factor 1α [Dissertation]. Jakarta: Universitas Indonesia; 2012.
23. Farhan FS, Masengi AF, Herawati M, Mulyawan W, Jusman SWA, Sadikin M. Effect of hypobaric hypoxia induction on cognitive function and glutamate receptor in sprague-dawley rats. eJournal Kedokteran Indonesia. 2019; 6(3): 201-6.
24. Fitriana N, Iswanti FC, Sadikin M. The role of hypoxia-inducible factor in mycobacterium tuberculosis-infected macrophages. Mol Cell Biomed Sci. 2024; 8(1): 23-30.
25. Irfannuddin I. Metabolisme oksidatif dan peranan neuroglobin terhadap homeostasis oksigen di otak. Sriwijaya Journal of Medicine. 2019; 2(3): 211-20.
26. Avivi A, Gerlach F, Joel A, Reuss S, Burmester T, Nevo E, *et al*. Neuroglobin, cytoglobin, and myoglobin contribute to hypoxia adaptation of the subterranean mole rat Spalax. Proc Natl Acad Sci. 2010; 107(50): 21570-5.
27. Mudjihartini N, Saekhu M, Jusman SWA, Sadikin M. Kadar neuroglobin dan sitoglobin dalam plasma, cairan serebro spinalis, dan jaringan otak pasien strok hemoragik. Muhammadiyah Journal of Geriatric. 2022; 3(1): 1-8.
28. Sisein EA. Biochemistry of free radicals and antioxidants. Sch Acad J Biosci. 2014; 2(2): 110-8.
29. Poyton RO, Ball KA, Castello PR. Mitochondrial generation of free radicals and hypoxic signaling. Trends Endocrinol Metab. 2009; 20(7): 332-40.
30. Pacher P, Nivorozhkin A, Szabó C. Therapeutic effects of xanthine oxidase inhibitors: renaissance half a century after the discovery of allopurinol. Pharmacol Rev. 2006; 58(1): 87-114.
31. Jusman SA, Halim A. Oxidative stress in liver tissue of rat induced by chronic systemic hypoxia. Makara Journal of Health Research. 2010: 13(1): 34-8.
32. Catherine C, Ferdinal F. Pengaruh hipoksia sistemik kronik terhadap kadar malondialdehid (MDA) pada darah dan jaringan ginjal tikus Sprague Dawley. Tarumanagara Medical Journal. 2018; 1(1): 54-8.
33. Mudjihartini N, Widia S, Suyatna F, Sadikin M. Stres oksidatif otak tikus pada induksi hipoksia sistemik kronik. Neurona. 2017; 34(3): n.p.
34. Wardaya W, Mulyawan W, Jusman SWA, Sadikin M. Oxidative stress, hypoxia-inducible factor-1α, and nuclear factor-erythroid 2-related factor 2 in the hearts of rats exposed to intermittent hypobaric hypoxia. HAYATI J Biosci. 2024; 31(1): 39-47.