Oxidative Stress, Hypoxia-Inducible Factor-1α, and Nuclear Factor-Erythroid 2-Related Factor 2 in the Hearts of Rats Exposed to Intermittent Hypobaric Hypoxia

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ABSTRACT

Hypobaric hypoxia is situation that might occur to helicopter pilots in Indonesia who must fly at an altitude of more than 3,048 m such as in Papua. It can be dangerous because hypoxic condition can affect person's performance. So far, the heart is known as an aerobic organ and very sensitive to hypoxic conditions. Hitherto, the effects of hypobaric hypoxia exposure on biomolecular aspects of the heart are still unclear. Therefore, this study assessed cardiac response in rats exposed to intermittent hypobaric hypoxia (IHH) (equivalent to 3,048 meters/10,000 feet). Sprague-Dawley rats were divided into six groups: control; acute hypobaric hypoxia (AHH); and IHH, for 7; 14; 21; and 28 days. We measured super oxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) activities, reduced glutathione (GSH), malondialdehyde (MDA), cytoglobin, myoglobin, HIF-1α, and Nrf2 level as our parameters. Activities of SOD, CAT, GPx and GSH increased while the levels of MDA, cytoglobin, myoglobin, HIF-1α, and Nrf2 decreased in all IHH groups compared with the AHH group. A biphasic pattern was observed as IHH sessions increased from 14 to 21 or 28. Where the IHH treatment for more than 14 sessions caused a decrease in endogenous antioxidants, but the response to hypoxia and oxidative stress increased. Our findings presented the molecular alterations of cardiac rats exposed to intermittent hypobaric hypoxia.

1. Introduction

It is well known that high-altitude exposure can induce a hypobaric hypoxia condition due to a decrease in barometric and oxygen partial pressures. Hypoxia situation can affect a person's performance. If this happens to a person responsible for the safety of others, such as an airplane pilot, it could put passengers at risk. Most commercial lines fly at altitudes of 10,000 meters and each aircraft is equipped with a pressurized cabin. Meanwhile, short-range air transport and helicopters are only allowed to fly at a maximum altitude of 3,048 meters and are not equipped with pressurized cabins. Nevertheless, the pilot of both types of altitude may suddenly be exposed to hypoxia situation and the situation can slowdown the reaction time of the pilot. It is known in aviation physiology some periodical short-term hypoxia can repair the reaction of the subject to normal or near normal (Gradwell and Rainford 2006; Davis *et al.* 2008; ICAO 2010).

The harmful effects of hypoxia can be experienced by helicopter pilots operating at high altitudes, since the helicopter cabin is not a pressurized cabin and is not equipped with oxygen apparatus. Altitudes up to 3,048 m (10,000 ft) are recognized in aviation as a physiological zone, since the impact of hypoxia on cognitive and psychomotor performance of pilots is relatively small and has little impact on flight safety.

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However, based on research by the Australian Army, 87% of non-pilot aircrews and 61% of pilots flying at altitudes up to 3,048 m may suffer from one or more symptoms of hypoxia (Grocott *et al.* 2007; Steinman *et al.* 2017; Dewi *et al.* 2021a, 2021b; Pham *et al.* 2021; Steinman *et al.* 2021).

In Papua, Indonesia, pilots often fly at altitudes of over 3,048 meters due to the very high mountains in this area. A 650-kilometer-long high mountain range runs through the center of Papua. The Jayawijaya Mountains are one example of this, which are renowned for having three of the highest peaks namely Jayawijaya (5,030 m/15,090 ft), Trikora (5,160 m/15,480 ft), and Yamin (5,100 m) (15,300 ft) (Sucipta *et al.* 2018). Consequently, pilots are frequently subjected to low partial oxygen levels and a hypobaric hypoxia condition during flights in Papua's mountainous areas. When the body is exposed to hypoxic circumstances, it will respond in an adaptive manner by providing both systemic and cellular responses to fulfill its oxygen needs (Herawati *et al.* 2017).

Hypoxia induced by high altitude exposure is known to increase ROS and reactive nitrogen species (RNS) levels and thus disrupt antioxidant defenses. Indeed, it has been shown that both acute and chronic hypoxic exposures increase oxidative stress. While increased oxidative stress has been found in response to both normobaric and hypobaric hypoxia, current studies indicate that hypobaric hypoxia may generate greater levels of oxidative stress than normobaric hypoxia (Debevec *et al.* 2017). ROS and RNS are balanced by a variety of endogenous antioxidants, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH) to maintain redox equilibrium in the cell (Ma 2013). The most important molecule to overcome hypoxia is hypoxia inducible factor-1α (HIF-1α), a transcription factor, which regulates the expression of an enzymes and the great number of proteins, such as cytoglobin (Cygb) that are needed to cope with hypoxia. Cytoglobin was recognized as the fourth type of globin expressed in mammals, primarily in fibroblasts, smooth muscle, and related cell types in various organs and tissues. It is structurally like myoglobin (Mb) and hemoglobin (Hb), and it is up regulated in tissues during hypoxia. It has been suggested that it may have a role in protecting against oxidative stress via ROS scavenger (Jusman *et al.* 2014; Zweier *et al.* 2021). Another

transcriptional factor involved in cellular responses to oxidative stress is nuclear erythroid 2-related factor 2 (Nrf2). It has been extensively studied in relation to diseases because Nrf2 is essential for regulating a wide variety of antioxidant enzymes involved in the detoxification and removal of oxidative stress (Ngo and Duennwald 2022).

The body's most aerobic organs, the heart and brain, need a lot of oxygen to function properly. The heart, on the other hand, is the only organ that must be aerobic and consumes the most oxygen. Heart muscle cannot produce enough energy to keep the heart healthy and functioning in hypoxic conditions (Herawati *et al.* 2017). The aim of this study is to analyze the effect of periodical, short term intermittent hypobaric hypoxia (IHH) on organ adaptation, in this case the heart, at the molecular level. To obtain more comprehensive information on the effect of IHH on the heart, we assess the oxidative stress status by evaluating the SOD, GPx, CAT, HIF-1α, Mb, Cygb, and malondialdehyde (MDA) as markers of lipid peroxidation. The Nrf2 measurement was also performed in this present study to verify the response of oxidative stress in the heart of a rat exposed to IHH. We used 523 mmHg air pressure in this study, which is equal to 3,048 meters of altitude, since we aimed to simulate the real situation experienced by many helicopter pilots, such as in Papua.

2. Materials and Methods

2.1. Ethical Approval

The experiments were performed in the Indonesian Air Force Institute of Aviation Medicine (Lembaga Kesehatan Penerbangan dan Ruang Angkasa, Lakespra) dr. Saryanto and Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia. All procedures were approved by the scientific ethics committee of the Faculty of Medicine, Universitas Indonesia (KET-943/UN2.F1/ETIK/PPM.00.02/2021).

2.2. Animals and Hypoxia Treatment

Thirty male Sprague-Dawley rats (200-250 g), age 2-3 month were obtained from The Indonesian Food and Drug Authority (BPOM RI) and divided into six groups. The control group was kept under ambient atmosphere and was not exposed to IHH at any point. The other groups were placed in a human hypobaric training chamber adjusted for animal studies and

received IHH treatment simulating an altitude of 3,048 m 1 h per day. The groups were exposed to one (1x; acute hypobaric hypoxia), seven (7x), fourteen (14x), twenty-one (21x), or twenty-eight (28x) IHH sessions. Animals were euthanized with ketamine + xylazine (50 mg/kg + 5 mg/kg) and had their hearts collected and stored at -20°C.

2.3. Tissue Homogenization

A piece (~100 mg) of heart tissue was homogenized in 1 ml of phosphate buffered saline (PBS) (0.01 M, pH 7.4) with tissue homogenizer. The homogenate was centrifuged at 5,000 × g for 5 min. The supernatant was collected and stored at -20°C until used in the analyses.

2.4. Antioxidants and Oxidative Stress Marker Measurement

The activities of SOD and GPX were assessed using commercial kits, RANSOD and RANSEL (Randox Laboratories Ltd., Crumlin, UK), respectively (Dewi *et al.* 2021a; Ramadhani *et al.* 2021). Catalase activity was measured by monitoring the consumption of hydrogen peroxide (H_2O_2) spectrophotometrically at 240 nm (Dewi *et al.* 2021a). Meanwhile, the levels of GSH were measured (Ekawati *et al.* 2016) by monitoring the interaction between the thiol reagent 5,5-dithiobis 2-nitrobenzoic acid (DTNB) and GSH, producing 5-thionitrobenzoic acid (TNB), whose levels can be read spectrophotometrically at 412 nm. Oxidative stress marker was assessed by examining lipid peroxidation product, which is MDA. Here, MDA levels were measured using the traditional thiobarbituric acid reactive substances (TBARS), according to Wills method (Dewi *et al.* 2021a).

2.5. Cytoglobin, Myoglobin, HIF-1α, and Nrf2 Measurements

The Cygb, Mb, HIF-1α, and Nrf2 concentrations were measured using sandwich ELISA kits for rat (MyBiosource, San Diego, USA; Elabscience, Texas, USA) following the manufacturer's protocol. All the data were divided by the total protein concentration in each sample, and the values are expressed as ng or pg/mg of protein. As explained previously, the total protein concentration was measured using absorbance at 280 nm (Ramadhani *et al.* 2021).

2.6. Statistical Analyses

All data were presented as the mean ± standard deviation (SD). For comparison among multiple groups of different times of exposure to IHH, a one-way analysis of variance (ANOVA) was used, followed by a Tukey's post-hoc test. A non-parametric test was applied when the data are not homogenous. Pearson correlation tests were also used to assess correlations between all measured variables. Statistical Package for Social Sciences (SPSS) software (IBM, USA) for Windows version 22.0 was used to analyze the data.

3. Results

3.1. SOD, GPx, and CAT Activities

Cardiac SOD activity increased after 7 sessions of IHH and reached its peak at 14x IHH exposure (Figure 1A). Compared with the acute hypobaric hypoxia (AHH) group, SOD activity increased by 48.25%; 67.19%; 51.61%; and 47.62% in 7x; 14x; 21x; and 28x IHH groups, respectively. Overall, SOD activity was higher in all IHH groups than in the AHH group. Similar findings were observed for GPx and CAT activities. Compared with the AHH group, GPx activity increased by 96.27%; 61.85%; 64.53%; and 69.31% in 7x; 14x; 21x; and 28x IHH groups, respectively (Figure 1B). In the case of CAT, the activities increased by 130.07%; 139.40%; 71.45%; and 67.97% in 7x; 14x; 21x; and 28x IHH groups, respectively, when compared with the AHH group (Figure 1C). Like SOD, CAT activity was the highest in rats exposed to 14 sessions of IHH.

3.2. GSH and MDA Levels

The highest level of GSH in the heart of rats exposed to hypobaric hypoxia was detected in the group treated for 14 days (Figure 2A). GSH concentration increased by 24.78%; 399.14%; 271.79%; and 238.46% in 7x; 14x; 21x; and 28x IHH groups, respectively, when compared with the AHH group. In the case MDA, the levels first increased in the AHH group and then decreased as the number of sessions increased (Figure 2B). MDA reached its lowest level in the 21x IHH group. Cardiac MDA concentration decreased by 47.51%; 66.61%; 76.82%; and 68.35% in 7x; 14x; 21x; and 28x IHH groups compared with the AHH group.

3.3. Cytoglobin, Myoglobin, HIF-1α, and Nrf2

Compared with the AHH group, Cygb levels tended to decrease as the number of sessions increased, reaching the lowest level after 14 IHH sessions (Figure 3A). Cygb levels decreased by 31.10%; 49.76%; 45.81%; and 24.61% in 7x; 14x; 21x; and 28x IHH groups, respectively, compared with that
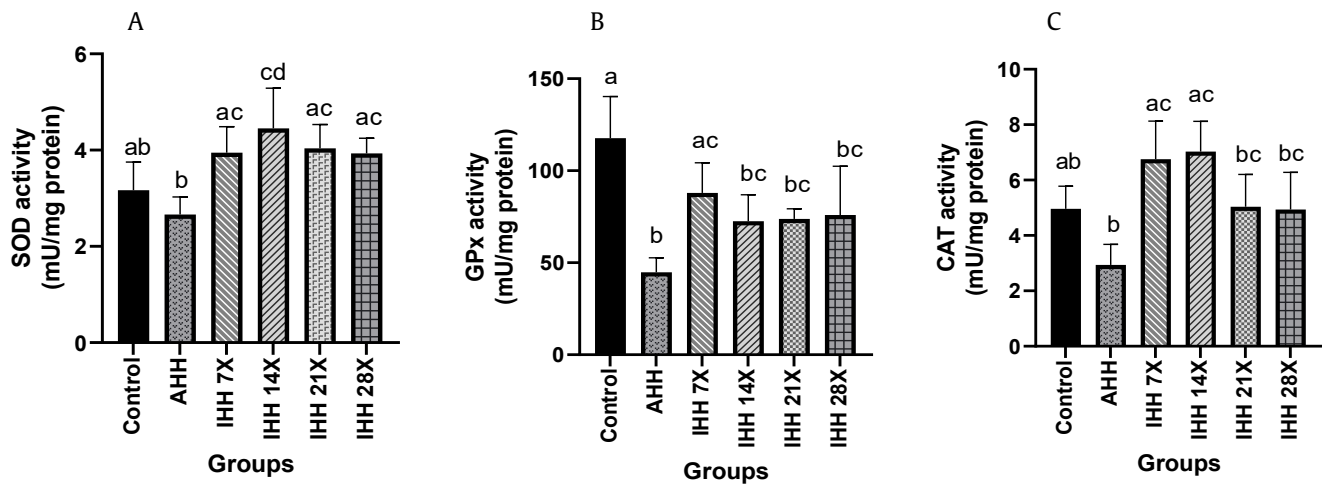
Figure 1. (A) Superoxide dismutase (SOD), (B) glutathione peroxidase (GPX), and (C) catalase (CAT). Activities in the heart of rats exposed to intermittent hypobaric hypoxia (IHH) simulating an altitude of 3,048 m (10,000 ft) for 1 h a day, once (acute hypobaric hypoxia, AHH), as well as for seven (IHH 7×), fourteen (IHH 14×), twenty-one (IHH 21×), and twenty-eight (IHH 28×) days. A group of rats never exposed to hypoxia was used as control. Groups that do not share the same letters are significantly different from each other (p<0.05)
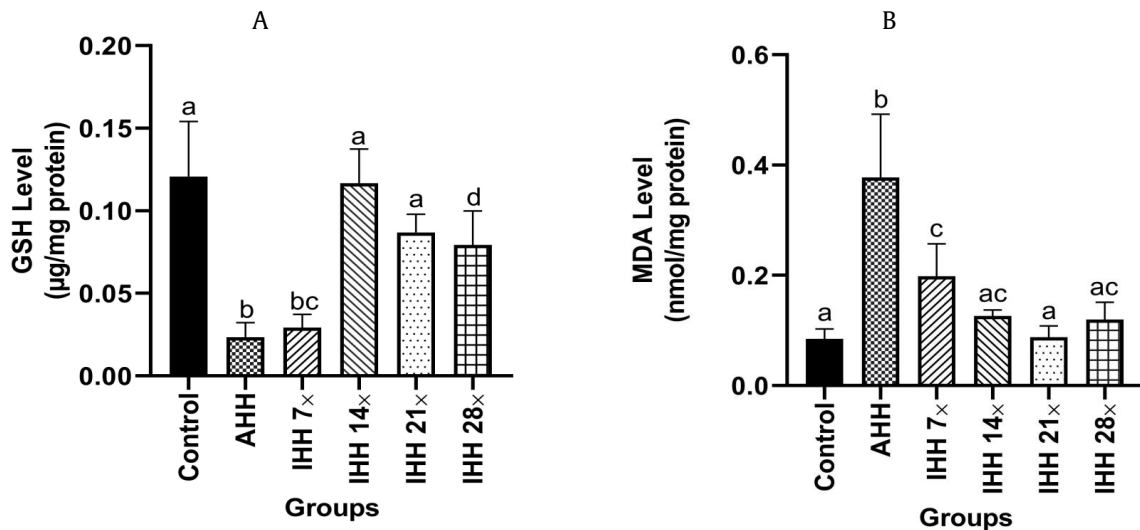

Figure 2. (A) Levels of glutathione (GSH), and (B) malondialdehyde (MDA). In the heart of rats exposed to intermittent hypobaric hypoxia (IHH) simulating an altitude of 3,048 m (10,000 ft) for 1 h a day, once (acute hypobaric hypoxia, AHH), as well as for seven (IHH 7×), fourteen (IHH 14×), twenty-one (IHH 21×), and twenty-eight (IHH 28×) days. A group of rats never exposed to hypoxia was used as control. Groups that do not share the same letters are significantly different from each other (p<0.05)

in the AHH group. Similar to Cygb, the lowest level of Mb was also found in the 14x IHH group (Figure 3B). Mb protein concentration decreased by 31.10%; 49.76%; 45.81%; and 24.61% in 7x; 14x; 21x; and 28x IHH groups, respectively, when compared with that in the AHH group. HIF-1α and Nrf2 protein levels roughly followed the same U-shaped curve of Cygb and Mb (Figures 3C and D), reaching the lowest levels

in the 14x IHH and 21x IHH groups respectively. HIF-1α levels decreased by 48.50%; 55.52%; 48.84%; and 37.76% in 7x; 14x; 21x; and 28x IHH groups, respectively, compared with the AHH group (Figure 3C). Nrf2 levels decreased by 33.76%; 52.38%; 56.74%; and 37.16% in 7x; 14x; 21x ;and 28x IHH groups, respectively, compared with the AHH group (Figure 3D).

3.4. Correlations

Pearson correlation test (Table 1) revealed positive correlation between MDA, Cygb, Mb, HIF-1α, and Nrf2. The results showed significant positive correlation between MDA with Mb (p = 0.002), MDA with HIF-1α (p = 0.000), and MDA with Nrf2 (p = 0.000). MDA also showed positive correlation with Cygb, although is not statistically significant (p = 0.063) (Table 1). The results also revealed that HIF-1α has significant positive correlation with Cygb

Table 1. Correlation coefficient matrix between MDA, Cygb, Mb, HIF-1α, and Nrf2 of cardiac rats exposed by intermittent hypobaric hypoxia

Variables	MDA	Cygb	Mb	HIF-1α	Nrf2
MDA	1				
Cygb	0.343	1			
Mb	0.542**	0.572**	1		
HIF-1α	0.794**	0.501**	0.524**	1	
Nrf2	0.748**	0.511**	0.546**	0.688**	1

*Correlation is significant at the 0.05 level (2-tailed)
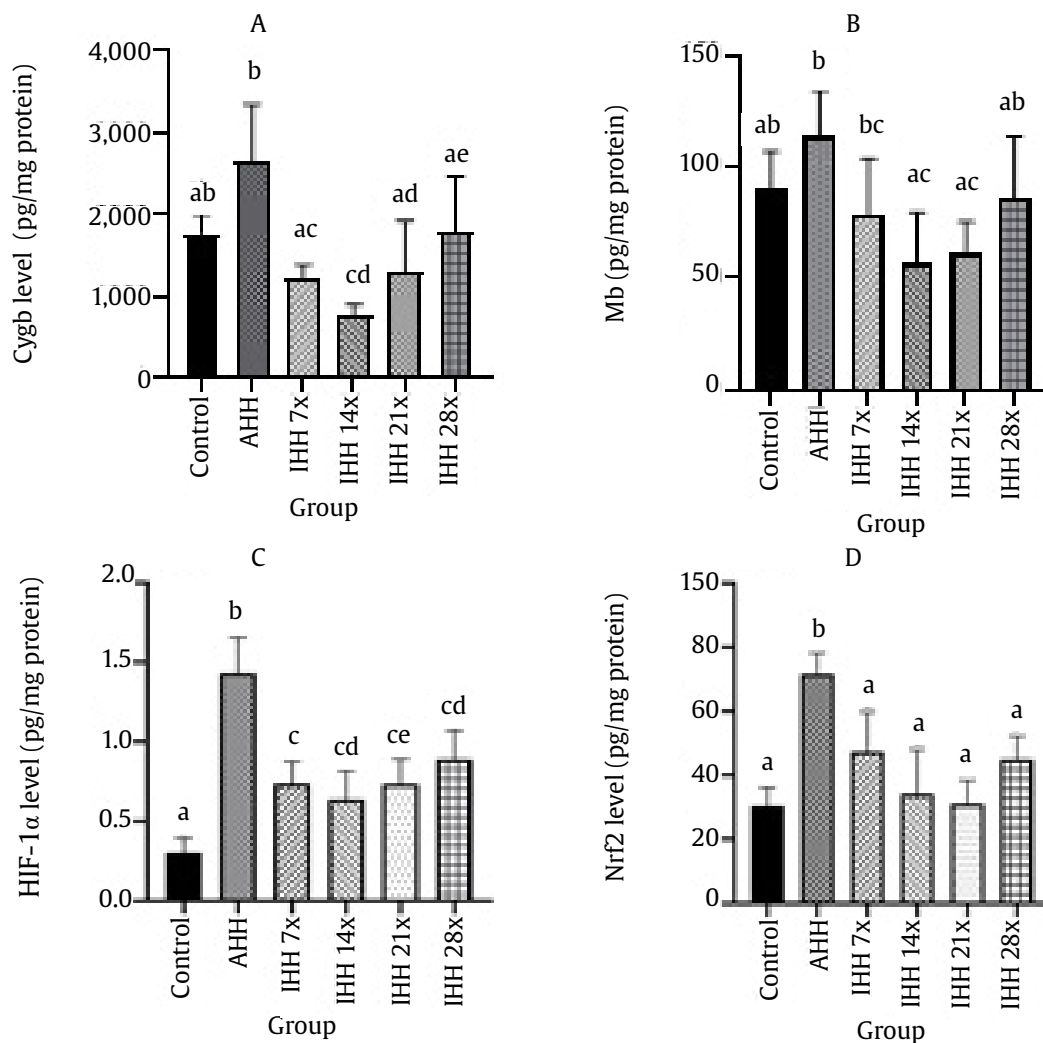**Correlation is significant at the 0.01 level (2-tailed)

Figure 3. (A) Cytoglobin (Cygb), (B) myoglobin (Mb), (C) hypoxia-inducible factor-1α (HIF-1α), and (D) nuclear factor erythroid 2-related factor 2 (Nrf2). Protein levels in the heart of rats exposed to intermittent hypobaric hypoxia (IHH) simulating an altitude of 3,048 m (10,000 ft) for 1 h a day, once (acute hypobaric hypoxia, AHH), as well as for seven (IHH 7×), fourteen (IHH 14×), twenty-one (IHH 21×), and twenty-eight (IHH 28×) days. A group of rats never exposed to hypoxia was used as control. Groups that do not share the same letters are significantly different from each other (p<0.05)

(p = 0.005), Mb (p = 0.003), and Nrf2 (p = 0.000). On the other hand, Nrf2 was found to have significant positive correlation Cygb (p = 0.004), and Mb (p = 0.001).

4. Discussion

In the present study, rats were exposed to hypobaric hypoxia simulating an altitude of 3,048 m (10,000 ft) for 1 h. This altitude was chosen based on the general view that at 3,048 m (the so-called physiological altitude in aviation medicine) flight capability remains unaffected (Gonchar and Mankovska 2010). However, even exposure to altitudes below 3,048 m in a depressurized chamber can elicit error proneness, slower reactions, physical fatigue, cognitive problems, and poor concentration (Chiang *et al.* 2021). Our hypobaric hypoxia treatment protocol can be classified as sub-chronic IHH (SCIHH), since the animals were exposed for 1 h to hypobaric hypoxia daily. This contrasts with chronic IHH (CIHH), which is defined by daily exposure to a sustained period (4–8 h) of hypobaric hypoxia. Repeating such stimuli over several days provides intermittent stimulation, which accurately represents physiological adaptation to high altitude (Smith 2007). In a previous study, we used IHH exposure with different air pressures, which were equivalent to those experienced at 18,000–35,000 ft and a one-week interval between repeated exposures to IHH (Herawati *et al.* 2017; Dewi *et al.* 2021a). Here, we exposed rats to IHH every day to obtain a more representative CIHH situation. For comparison purposes, we used rats exposed to AHH and rats never exposed to hypoxia.

The International Civil Aviation Organization (ICAO) Standards and Recommended Practices (SARPS) provides guidelines for the provision and use of supplemental or emergency oxygen systems (ICAO 2010). The guidelines clearly state that all crew members and 10% of passengers in a flight operating at flight altitudes at which the atmospheric pressure between 10,000 ft (700 hPa) and 13,000 ft (620 hPa) with a duration of more than 30 min need oxygen supply. Conversely, pilots in Indonesia, particularly in Papua, often forget to use the oxygen supply equipment when they need to reach ≥ 10,000 ft. Although most commercial flights in Papua fly below 10,000 ft, they frequently need to fly above 10,000 ft or higher because of the mountainous

terrain. Thus, in-flight hypoxia symptoms are likely to be experienced by these pilots, including a significant risk of unconsciousness for pilots who had not received hypobaric-awareness training. Currently, refresher hypobaric-awareness training courses in chambers that simulate high-altitude environments are not mandatory in Indonesia. Thus, we investigated molecular events associated with acute HH exposure, as well as repeated exposure to HH, simulating 10,000 ft.

Our findings revealed that acute exposure to pressure experienced at 10,000 ft, which is normally considered as safe, does induce significant decreases in level of SOD, GPx, and GSH. Although not statistically significant, CAT activity also decreased in this group. This finding agrees with previous studies that showed an increase in ROS production induced by HH exposure (Bailey *et al.* 2009; Faoro *et al.* 2011; Strapazzon *et al.* 2016). Decreased levels of SOD, GPx, CAT, and GSH in this study are possibly the result of the consumption of these enzymatic and non-enzymatic antioxidants by excess ROS produced in response to AHH. Accordingly, lipid peroxidation levels greatly increased in the AHH group compared with the control group. This finding confirmed that acute exposure to 10,000 ft increases ROS levels and induces oxidative stress due to a significant imbalance between ROS and antioxidants, which subsequently leads to oxidative damage to lipids, as evidenced by a higher MDA level. Noteworthy, MDA levels were positively correlated with Cygb, Mb and HIF-1α levels. Increased MDA levels and decreased antioxidant levels were associated with an increase in HIF-1α protein concentration after AHH exposure at 10,000 ft.

Similar to our previous findings, we detected a significant positive correlation between Mb and HIF-1α in the heart of rats exposed to hypoxia (Herawati *et al.* 2017). Myoglobin facilitates oxygen transport, stores oxygen, and scavenges nitric oxide within myocytes (Garry *et al.* 2003). Although chronic hypoxia promotes a set of HIF-1α mediated adaptations (e.g., angiogenesis, erythropoiesis, cellular proliferation, vascular remodeling, and glycolytic metabolism), there is no evidence confirming that myoglobin is a direct downstream target of HIF-1α (Kanatous *et al.* 2009). Based on our present and previous findings, it seems that Mb expression might be under the regulation of HIF-1α, especially under hypoxia. One possible mechanism

underlying this relationship is the fact that HIF-1α regulates transferrin expression, which, in turn, plays a key role in the formation of Mb (Herawati *et al.* 2017). Prolonged hypoxia upregulates Mb (mRNA and protein levels) in breast cancer cells, which was partially attributed to the transcriptional activation of hypoxia-inducible factors-1 and -2 (HIF-1, HIF-2) (Kristiansen *et al.* 2011). Other studies, however, reported either no change or a decrease in Mb levels in animals and humans subjected to varying degrees of hypoxia (Terrados *et al.* 1986; Armstrong *et al.* 1992).

Our present study also revealed the possibility of an adaptation phenomenon, as the number of sessions increased, in the hearts of rats exposed to IHH. Two weeks of daily hypobaric hypoxia exposure (14x IHH) seemed to be the optimal to induce adaptation to the hypoxia. After 14 sessions of hypobaric hypoxia, the levels of cardiac SOD, CAT, and GSH activities reached maximum values, whereas the levels of cardiac Cygb, Mb, and HIF-1α reached minimum levels. The higher levels of antioxidants in the 14x IHH group indicate that the initial stress caused by AHH had been overcome and the heart of rats, at this point, had better capacity to deal with oxidative stress with a higher antioxidant capacity. When the number of sessions increased to 21 and 28, however, no further increase in antioxidant levels were observed. At this moment, we cannot explain the mechanism behind this phenomenon, which warrants further research.

Pearson correlation analyses revealed that positive correlation between Cygb and MDA, although is not statistically significant. Despite efforts in understanding the biological role of Cygb, its exact function remains unclear (Ukeri *et al.* 2022). Studies indicate that Cygb is upregulated in response to cellular hypoxia/ischemia and suggest a possible role of Cygb in oxygen sensing and nitric oxide (NO) homeostasis (Shaw *et al.* 2009; Avivi *et al.* 2010; Emara *et al.* 2010). Accordingly, we showed here that rats exposed to acute hypobaric hypoxic have higher levels of cardiac Cygb. Our findings showed the significant positive correlation between Cygb and HIF-1α expression. This results was fit with previous study that revealed Cygb was regulated by HIF-1α (Jusman *et al.* 2014). HIF-1α levels positively correlated with Nrf2. As mentioned above, hypoxia induces oxidative stress and consequently decreases antioxidant levels since they are used to scavenge excess ROS. Similar to HIF-1α Nrf2 concentration also

peaked in the AHH group, but it then decreased to its nadir at 21x IHH. Upregulation of Nrf2 in the AHH group indicates oxidative stress-induced activation of this transcription factor, which is known to be activated in response to oxidative and electrophile stresses arising from endogenously generated ROS (Malec *et al.* 2010). The present results, thus, support this canonical mechanism of Nrf2 activation.

Overall, our findings indicated that repeated exposure to hypobaric hypoxia corresponding to 10,000 ft might have beneficial adaptive effects. However, there is a biphasic pattern of such effects, in which exposure for more than 14 sessions results in decreased SOD, CAT, and GSH levels. Noteworthy, increased levels of MDA, Cygb, Mb, HIF-1α, and Nrf2 were observed in the heart of rats after two weeks of daily exposure to hypobaric hypoxia. Molecular alteration such as increased levels of MDA and expression of HIF-1α followed by decreased activity of SOD and GSH were also found in the heart of mice exposed to hypobaric hypoxic conditions (7,000 m) for 23 hours compared to control (Li *et al.* 2020). In addition, the hypobaric hypoxic condition also widens the myocardial space and cause interstitial capillary hyperemia (Li *et al.* 2020).

Besides the changes in the cardiac molecular aspects shown in this study, several studies have shown that prolonged exposure to hypobaric hypoxia can cause organ and tissue changes, such as in the urogenital systems. Continuous exposure to hypobaric hypoxia (25,000 ft) for up to 7 days caused functional changes with significant structural damage in the rat kidneys (Chhabra *et al.* 2018). Sustained hypobaric hypoxia causes excessive expression of HIF-1α that induce an inflammatory and profibrotic pathways resulting in apoptosis in kidney tissue (Chhabra *et al.* 2018). Another study showed that exposure to acute hypobaric hypoxia for 48 hours caused an increase in MDA and ROS levels, followed by a decrease in GSH levels in renal rat (Rathi *et al.* 2023). That study also showed tissue damage and impaired kidney function in rats exposed to acute hypobaric hypoxia (Rathi *et al.* 2023).

Taken together, the data presented here emphasize the importance of hypobaric awareness training courses for commercial air-flight pilots in Indonesia, given the observed molecular alterations under hypobaric hypoxia. This is especially relevant for the mountainous Papua region. Examples to be followed include several countries, such as Saudi

Arabia, New Zealand and the United States, where every 3–5 years pilots are required to complete hypobaric awareness training courses (Neuhaus and Hinkelbein 2014). Lastly, our results show that even inside the so-called physiological altitude molecular alterations are observed in the heart and, therefore, hypoxia-related symptoms at 3,048 m (10,000 ft) should be taken seriously.

Acknowledgements

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