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Malondialdehyde and carbonyl levels in skeletal muscle tissues after intermittent hypobaric hypoxia exposures

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ABSTRACT

Background: Hypobaric hypoxia is a state of decreased oxygen pressure at high altitudes that can lead to hypoxia and oxidative stress as a result. Skeletal muscle is one of the important organs that can be affected by oxidative stress and cause contractile dysfunction.

Objective: This study aimed to evaluate the impact of intermittent hypobaric hypoxia on oxidative stress markers in rat skeletal muscle, by measuring malondialdehyde (MDA) and carbonyl levels.

Methods: Twenty-five Wistar rats were allocated into five groups, including one control group and four hypoxic groups (I-IV). The hypoxic groups were exposed to an altitude of 25,000 feet for 5 minutes using hypobaric chamber in once (I), twice (II), three (III), and four (IV) times, with a 7-day interval period between exposures. The control group remained in normobaric conditions throughout the study. MDA levels were measured by thiobarbituric acid (TBA) test, while carbonyl levels were measured using 2,4-dinitrophenylhydrazine (DNPH) reagent.

Results: The MDA level was significantly increased in group I compared to the control group (p=0.008). There were decreasing MDA levels in groups II, III, and IV compared to group I. The carbonyl level was significantly higher in group I than the control group (p=0.000), with an even higher level observed in group II. Although the carbonyl levels tended to decrease in groups III and IV, they still remained higher than those of the control group.

Conclusion: Exposure to hypobaric hypoxia leads to an increase in MDA and carbonyl levels in the skeletal muscles, indicating an elevation of oxidative stress levels. However, the subsequent intermittent hypobaric hypoxia exposure resulted in a reduction in these levels, implying that skeletal muscles may adapt to hypoxic conditions.

Keywords: carbonyl, hypobaric hypoxia, malondialdehyde, skeletal muscle

Introduction

Due to lower atmospheric pressure at higher heights, oxygen partial pressure decreases. Individuals who are habituated to living at sea level and abruptly move to a high altitude can experience acute pathophysiological changes in their bodies. This could be caused by hypobaric hypoxia, a hypoxic condition when the partial oxygen pressure is low at a high altitude. Some symptoms that can occur after exposure to hypobaric hypoxia are poor concentration, impaired cognitive function, visual disturbances, hot flashes, and numbness [1]. In the long run, people residing at high altitudes tend to undergo an adaptation process in their physiological functions [2].

Oxygen is an indispensable molecule that is required by the body for the energy production process through the electron transport chain/oxidative phosphorylation in the mitochondria. Atmospheric pressure will decrease linearly with altitude increase, decreasing the inspired oxygen pressure. At an altitude of 5000 m or 8000 m above sea level, the

inspired oxygen pressure will decrease to 56.2% or 38.3% from the normal value, respectively [3]. Decreasing the inspired oxygen pressure reduces the driving pressure for gas exchange in the lung which affects the mitochondrial level, where the electron transport chain occurs and oxygen is the final electron acceptor. Disruption of oxidative phosphorylation reactions in mitochondria will lead to increased production of superoxide anions in complexes I and III, due to electron leakage in these complexes and reaction with oxygen [4,5].

Increasing superoxide anion production, which is one of the reactive oxygen species (ROS), will cause oxidative stress and can cause various modifications of macromolecules in the body such as DNA, carbohydrates, proteins, and lipids, which are associated with cell metabolism disturbances and cell death induction. ROS can attack lipid and protein molecules, and produce lipid peroxidation and protein oxidation. Lipid peroxidation reaction can be measured through malondialdehyde (MDA) levels, while protein oxidation can be measured through carbonyl levels [6,7]. In biological tissues, these lipid hydroperoxides are broken down into various products including malondialdehyde (MDA) [8]. One of the MDA level measurements is the Wills method, which measures MDA by the addition of thiobarbituric acid (TBA) to form pink color and it is read by a spectrophotometer [9]. Excessive ROS can attack proteins and cause conformational changes that can eliminate their function. Protein carbonylation is a protein oxidation process due to excess ROS and produces carbonyls (ketones or aldehydes) [10]. These carbonyl compounds can form hydrazones when reacted with 2,4-dinitrophenylhydrazine (DNPH) [11].

One of the tissues that can be affected by hypoxia is the skeletal muscle. Skeletal muscle oxygen demand is very high, especially during exercise. When doing maximal physical activity, skeletal muscles are supplied the blood from 90% of cardiac output, to fulfill oxygen demand [12]. When oxygen is insufficient, the increase of metabolic function in active skeletal muscle will be accompanied by the hypoxia effect [13]. Hypoxic conditions in muscle will cause an increase in ROS and result in deleterious effects, such as reduced energy production and increased muscle atrophy [14]. Therefore, this study was conducted to analyze the effect of intermittent hypobaric hypoxia exposure on oxidative stress in skeletal muscle by measuring the MDA and carbonyl levels.

Methods

Experimental design

This study was an in vivo experimental study using Wistar rats (aged 6-8 weeks and weight 150-200 g), that has been conducted in the Laboratory of Biochemistry and Molecular Biology, Faculty of Medicine Universitas Indonesia and Lakespra TNI AU. Rats were purchased from the Research and Development Agency of the Indonesian Ministry of Health and adapted for one week before treatment, with eating and drinking ad libitum. Based on Federer's formula, the total rats used were twenty-five rats divided into 5 groups (n=5 each). Four groups were the treatment group which consisted of group I (exposed to a single episode of IHH), group II (exposed to two episodes of IHH), group III (exposed to three episodes of IHH), and group IV (exposed to four episodes of IHH). One group acted as the control group and the rats were not exposed to IHH. Hypobaric hypoxia exposures were created by placing rats into a hypobaric chamber simulated to an altitude gain of 25.000 feet and maintained for 5 minutes. Group II, III, and IV were exposed to multiple IHH episodes, and the treatment was taken in 7 days intervals between IHH exposure. After IHH treatment, rats were sacrificed and gastrocnemius muscles were collected. This study was approved by the ethical committee of the Faculty of Medicine Universitas Indonesia with no. KET-774/UN2.F1/ETIK/PPM.00.02/2020.

Tissue preparation

One hundred milligrams of muscle tissues were homogenized in 1 mL phosphate buffer saline (PBS) 0.01 M pH 7.4. The centrifugation was conducted at 3000 rpm for 10 min to obtain the supernatant

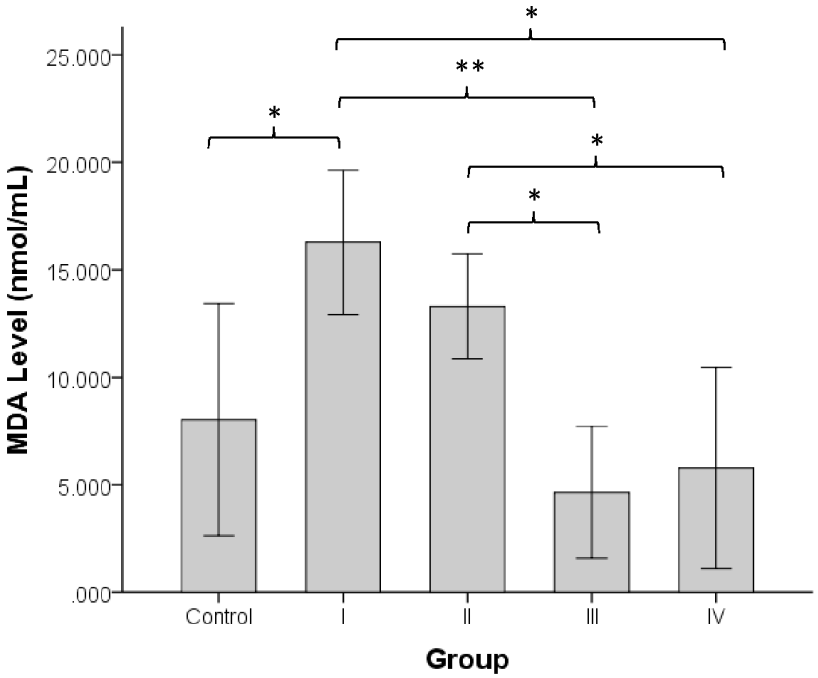

Figure 1. MDA level in rat muscle tissues after intermittent hypobaric hypoxia exposures. Control group: no exposure, group I: 1x exposure, group II: 2x exposures, group III: 3x exposures, group IV: 4x exposures. Significance *= p<0.05, **= p<0.001, One-way ANOVA test.

and remove the debris from the homogenate. This supernatant was used for the measurement of MDA and carbonyl levels.

Malondialdehyde level assay

We measured the MDA level using thiobarbituric acid (TBA), known as the Wills method. Thiobarbituric acid (TBA) could react with the MDA compound and create pink color. Trichloroacetic acid (TCA) 20% was added to the supernatant to make protein precipitation and remove the protein by centrifugation at 6000 rpm for 5 minutes. The supernatant was kept and added by TBA 0.67% then incubated at boiling water 95-100ºC. The pink color that arose from the reaction MDA with TBA was read by a spectrophotometer at 530 nm. We used 1,1,3,3-tetra ethoxy propane (TEP) with various concentrations (0.3125, 0.625, 1.25, 2.5, and 5 nmol/mL) to create a standard curve for MDA. The MDA level was calculated using the regression linear formula generated from the standard curve.

Carbonyl level assay

We measured the carbonyl level using 2,4-dinitrophenylhydrazine (DNPH) which could react with the carbonyl compound and generate a color [10]. The supernatant was added with 10 mM DNPH in 2.5 M HCl and incubated at room temperature in the dark. Trichloroacetic acid (TCA) 20% was added to the mixture and then incubated on ice for 5 minutes. After centrifugation of 10,000 g for 10 minutes, the pellet was formed, and discard the supernatant. The pellet was resuspended by ethanol: ethyl acetate solution (1:1). Then added guanidine HCl into the suspension and centrifuged at 10,000 g for 10 minutes, discarding the pellet. The color complex produced by this reaction was read by a spectrophotometer at wavelength 360-385 nm. The carbonyl level was obtained by multiplying the absorbance number with the extinction coefficient for carbonyl (22000 $M^{-1}cm^{-1}$).

Data analysis

Data were analyzed using SPSS software version 20. The mean differences in MDA and carbonyl between the treatment and control groups were tested with one-way ANOVA and continued with LSD post hoc analysis. Significance is indicated by a p-value <0.05.

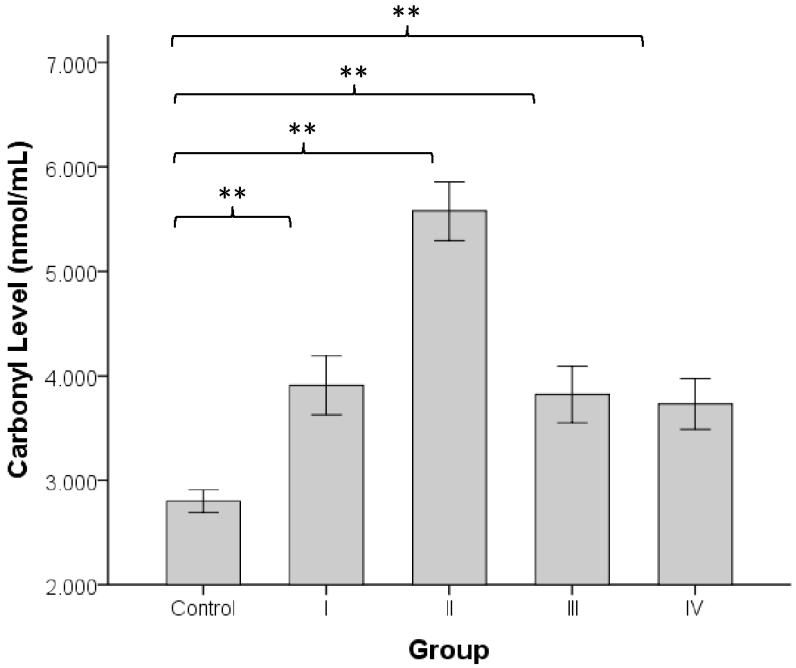

Figure 2. Carbonyl level in rat muscle tissues after intermittent hypobaric hypoxia exposures. Control group: no exposure, group I: 1x exposure, group II: 2x exposures, group III: 3x exposures, group IV: 4x exposures. Significance **=p<0.001, One-way ANOVA test.

Results

In this study, we found the MDA level in muscle tissues was increased in group I compared to the control group. After that, the MDA level in group II was decreased from that of group I but still higher compared to the control group (Figure 1). In groups III and IV, the MDA level was lower than in the control group. The one-way ANOVA test showed significance (p=0.002) and LSD post-hoc analysis found a significant difference between group I and the control group (p=0.008). There was a significantly decreased between group III compared to group I (p=0.000) and II (p=0.006), and also between group IV compared to group I (p=0.001) and II (p=0.014).

We demonstrated the carbonyl level in this study was increasing in groups I, II, III, and IV compared to the control group, but a high increasing found in group II (Figure 2). In groups III and IV, the carbonyl level was still higher than in the control group, but it decreased compared to group II. After statistical analysis using a one-way ANOVA test, there is a significant difference among all treatments (group I-IV) and the control group (p=0.000). In LSD post-hoc analysis found a significant difference between all treatment groups compared to the control group (p=0.000).

Discussion

In this study, we found the MDA and carbonyl levels in muscle tissues increased after acute hypobaric hypoxia exposure (group I) compared to the control group. This is supported by the previous study, Hou *et al* reported the increase of serum MDA levels in rats exposed to 24 hours of hypobaric hypoxia [15]. Chaudhary *et al*. reported increased levels of MDA in muscle tissue after exposure to chronic hypobaric hypoxia for 3 days, 7 days, 14 days, and 21 days. The increase of MDA levels in the gastrocnemius muscle was found to be higher than that of the soleus muscle [16]. The other study by Agrawal *et al*, which investigated protein modification in rat skeletal muscle after being exposed to hypobaric hypoxia for 6, 12, and 24 hours, reported that all treatment groups had higher levels of carbonyl compound than the control group (normoxic rat) [17].

After exposure to intermittent hypobaric hypoxia, muscle MDA levels in group II began to decrease compared to group 1 although the

levels were still higher than in the control group. In group III and IV, MDA levels decreased lower than in the control group. However, the results of muscle carbonyl levels in group II still significantly increased compared to group I and control, and carbonyl levels in group III and IV began to decrease near to control group. Muscle MDA and carbonyl levels tend to decrease after exposure to intermittent hypobaric hypoxia, indicating an adaptation mechanism of muscle tissue to hypobaric hypoxic conditions. Previous studies have also shown a decrease in carbonyl and MDA levels in rat brains after intermittent hypobaric hypoxia exposure, supported by increasing antioxidant enzyme activities such as superoxide dismutase (SOD) and catalase in those tissues [18]. SOD is an enzyme that catalyzes the conversion of anion superoxide ($O_2^{-\bullet}$) into oxygen (O_2) and hydrogen peroxide (H_2O_2). Whereas catalase is an enzyme that decomposes hydrogen peroxide (H_2O_2) into water (H_2O) and oxygen (O_2).

Acute hypobaric hypoxia conditions can increase the production of ROS molecules that cause oxidative stress. The occurrence of oxidative stress in hypoxic tissues can occur through several mechanisms, such as leakage of the electron transport chain in mitochondria, NADPH oxidase activity, activation of the xanthine oxidase pathway, and increased production of catecholamines [19]. The parameter usually used to measure oxidative stress is MDA, which is a product of lipid peroxidation, and carbonyl compounds due to the oxidation of protein molecules [8,10].

One mechanism of cellular adaptation to hypoxic conditions is the Hypoxia-Inducible Factor-1 (HIF-1) activity. HIF-1 is an important transcription factor that mediates the cellular response to lack of oxygen, by increasing vascularization to hypoxic areas and decreasing tissue oxygen consumption. The mechanism of decreasing tissue oxygen consumption is through the inhibition of peroxisome proliferator-activated receptor co-activator-1β (PGC-1β) which plays a role in the proliferation of type I muscle fibers. Hypoxia will cause a decrease in mitochondria-rich type I muscle fibers which have a fatigue-resistant characteristic [20]. In addition, intermittent hypoxia causes an increase in type II muscle fibers that have fewer mitochondria than type I [21]. A reduction number of type I muscle fibers and an elevation of type II muscle fibers have an impact on increasing the anaerobic metabolic capacity of skeletal muscle. The reduction in muscle fibers that have many mitochondria, affects reducing ROS production under hypoxic conditions [20].

On the other hand, adaptation to hypoxic conditions can also be performed by increasing antioxidant levels. In rat brains, superoxide dismutase activity increased along with repeated exposure to hypobaric hypoxia as an adaptation mechanism [18]. Superoxide dismutase catalyzes the conversion of superoxide anion into hydrogen peroxide. Furthermore, hydrogen peroxide can be reduced to water by catalase, glutathione peroxidase, or thioredoxin-dependent peroxiredoxin. Acute hypoxia exposure also increases the antioxidant capacity of rat skeletal muscle through increased expression of nuclear factor erythroid 2-related factor 2 (Nrf2), that act as a transcription factor that binds to the antioxidant-response element (ARE) in the antioxidant genes [22].

The interesting one from this study is the carbonyl levels were still increased in group 2 (2 times exposures) while MDA levels had started to decrease in this group, which indicates that protein oxidation still occur after the second exposure while the lipid peroxidation seemed to decline. The muscle tissue suggests this contains a lot of protein so it has the potential to undergo protein oxidation/carbonylation [23,24]. The occurrence of protein carbonylation in skeletal muscle could cause a decrease in ATP production and induce muscle atrophy [14]. The limitation of this study, we did not measure the level or activity of antioxidant enzymes to confirm the adaptation toward oxidative stress in this experiment.

Conclusion

Exposure to hypobaric hypoxia causes an increase in oxidative stress in rat muscle tissue as proved by the elevation of MDA and carbonyl

levels. Intermittent hypobaric hypoxia exposure stimulates the adaptation process in muscle tissue to hypoxia, characterized by the reduction of MDA and carbonyl levels on repeated exposure.

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Author contributions

Study conception and design: SD and W; data collection: ARS and ARD; analysis and interpretation of results: SD and W; draft manuscript preparation: SD, ARS and ARD. All authors reviewed the results and approved the final version of the manuscript.

Declaration of interest

There is no conflict of interest in this study.

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