HAYATI Journal of Biosciences 24 (2017) 131–135

Contents lists available at ScienceDirect

HAYATI Journal of Biosciences

journal homepage: http://www.journals.elsevier.com/hayati-journal-of-biosciences

Expression of Hypoxia-Inducible Factor-1α and Myoglobin in Rat Heart as Adaptive Response to Intermittent Hypobaric Hypoxia Exposure

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ARTICLE INFO

Article history:
Received 29 December 2016
Received in revised form
13 July 2017
Accepted 23 August 2017
Available online 3 October 2017

KEYWORDS:
intermittent hypobaric hypoxia,
HIF-1α,
Mb

ABSTRACT

The aim of this study was to investigate the influence of intermittent hypobaric hypoxia on the expression hypoxia adaptation proteins, namely hypoxia inducibla factor-1a (HIF-1a) and myoglobin (Mb). Twenty five male Sprague-Dawley rats were exposed to intermittent hypobaric hypoxia in a hypobaric chamber in Indonesian Air Force Institute of Aviation Medicine, for 49.5 minutes at various low pressure, 1 week interval for 4 times (day 1, 8, 15 and 22). HIF-1α and Mb protein were measured with ELISA. mRNA expression of Mb was measured with one step real time RT-PCR. HIF-1α protein levels increased after induction of hypobaric hypoxia and continues to decrease after induction of intermittent hypobaric hypoxia 3 times (ANOVA, $p = 0.0437$). mRNA expression and protein of Mb increased after induction of hypobaric hypoxia and continues to decrease after induction of intermittent hypobaric hypoxia 3 times (ANOVA, $p = 0.0283$; 0.0170), and both are strongly correlated (Pearson, $r = 0.6307$). The heart of rats adapted to intermittent hypoxia conditions by upregulation the expression of HIF-1a and myoglobin and then both return to normal level.

1. Introduction

Hypoxia is a condition in which oxygen supply cannot fulfill the need of an organism, its organ, or even a cell. In this situation, the subject (organism, organ, or cell) has to activate various mechanisms to maintain the energy level. The hypoxia condition induces adaptation responses to defend homeostasis in the body. Physiologic responses such as increased heart, pulse, and respiratory rates increase oxygen supply. Transcription factor hypoxia-inducible factor (HIF)-1α will be stabilized with HIF-1β. This heterodimer regulates many of the genes in hypoxic condition. Altered cell metabolism, from aerobic to anaerobic, increases lactic acid production that makes acidosis. High blood pressure was recorded during hypoxia, to increase oxygen distribution through red blood cells (Loscalzo J, 2010).

In intermittent hypoxia, the subject is exposed to a certain low oxygen pressure frequently, and in an experiment, subject is exposed to it at a certain interval between each treatment. It can be presumed that any adaptation process would be repeated. Intermittent hypobaric hypoxia (IHH) is linked with oxidative stress, impairing cardiac function. However, early IHH also activates cardioprotective mechanisms (Herrera EA *et al.*, 2015). Clinical observation on preconditioning ischemia (Muray CE *et al.*, 1986) and previous experimental studies on the effect of

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Peer review under responsibility of Institut Pertanian Bogor.

https://doi.org/10.1016/j.hjb.2017.08.010

intermittent hypoxia on the brain (Mulyawan W, 2012) and heart (Hidayat A, 2011) suggest that this condition might be beneficial for the subject.

The HH is relatively rare in the usual condition. Most people live at the sea level to 500 m altitude, where the influence of atmospheric pressure is relatively nil. The hypoxic conditions at high altitudes present a challenge for survival, causing pressure for adaptation. Interestingly, many people living at high-altitude regions (particularly in the Andes) are maladapted, with a condition known as chronic mountain sickness (Zhou D *et al.*, 2013). Studies of Tibetan highlanders showed that polymorphisms in candidate genes show signatures of natural selection as well as well-replicated association signals for variation in hemoglobin levels. Studies of Ethiopian highlanders found that variants associated with the hemoglobin variation among Tibetans or other variants at the same loci do not influence the trait in Ethiopians (Aranburu GA *et al.*, 2012; Peng Y, *et al.*; 2011).

When the body undergoes hypoxic conditions, adaptively, body will provide systemic and cellular responses to meet the oxygen requirements. Semenza has mentioned that the systemic hypoxic conditions increased the expression of erythropoietin (EPO), which led to an increase in the number of red blood cells in the oxygen-carrying molecule (Semenza, 2004). In these studies, it is known that in the condition of hypoxia, HIF-1α is a transcription factor that regulates the increased expression of EPO.

Circulation of blood throughout the body is regulated through blood pressure, which is dependent on the heart pumping and blood flow. Exposure of mice to hypoxia leads to a significant rise in blood pressure (Edckardt *et al.*, 2005). This rise is associated with microvascular endothelial changes, subtle tubulointerstitial injury, inflammation, and interstitial cell proliferation (Johnson RJ *et al.*, 2002). Therefore, the heart selected as samples in this study.

However, besides people living in high-plateau regions such as Tibet (Peng Y *et al.*, 2011), Andes (Zhou D *et al.*, 2013), or Ethiopia (Aranburu GA *et al.*, 2012), pilots are prone to unpleasant or even the deleterious effect of hypoxia, resulting from sudden low atmospheric pressure. Sudden hypoxia will reduce the psychomotor activities (Gradwell DP, 2006), which is very dangerous for the pilot. Fortunately, the danger can be reduced by a special training in which the pilot is exposed to a hypoxic condition by placing him in a low-pressure hypoxic chamber. The air pressure is made similar to various altitudes which occur in aviation.

It is well known that the heart and brain are the most aerobic organs in the body, and therefore, both consume a great portion of respiratory oxygen. However, heart is an obligate aerobic organ that consumes more oxygen than any other organ. Heart muscle cannot produce enough energy to sustain cardiac function and viability under hypoxic conditions. In the absence of sufficient oxygen, electron transport chain ceases, and cardiac energy demands are not met (Giordano FJ, 2005).

Therefore, we wondered if the intermittent hypoxia would have beneficial effects on the heart, they would be indicated by hearts' HIF-1α and myoglobin (Mb) levels. We realized the observation on the rats placed in various conditions of low atmospheric pressure in the hypobaric chamber.

2. Material and Methods

2.1. Animal model and sample preparation

All the following experimental designs and procedures have been reviewed and approved by a Scientific Ethic Committee. This experimental animal study was conducted in the Indonesian Air Force Dr Saryanto Institute of Aviation Medicine (Lakespra Dr Saryanto: Lembaga Kedokteran Penerbangan dan Ruang Angkasa Dr Saryanto) and the Department of Biochemistry and Molecular Biology, Faculty of Medicine University of Indonesia from 2015 to 2016. Twenty-five male Sprague–Dawley rats (200–250 g) were obtained from the Institute of Health Research and Development, Jakarta, divided into five groups. The rats of the control group were placed in environment atmosphere without exposure to HH. The other groups were placed in human hypobaric training chamber according to the number of experiments carried out (for one, two, three, and four times). Hypoxia group was exposed to HH for one time and hypoxia preconditioning groups were exposed to HH for two (IHH 1×), three (IHH 2×), and four times (IHH 3×).

After placing the rats in the chamber, the pressure is lowered quickly (within 7 min) to a value that is equal to the air pressure at 35,000-feet altitude. After 1 min in this condition, the pressure was increased progressively to a pressure equal to the one at 30,000-feet altitude (during 3 min), to a pressure equal to that at 25,000-feet altitude (during 5 min), and then to a pressure equal to the one at 18,000-feet altitude (during 30 min). At the end of the last altitude, the conditions were made to a normal high pressure. The procedure is shown in Figure 1. The experiment was repeated for three times after the first exposure, with a 1-week interval between each experiment. At the end of each experiment, animals of the correspondent group were killed under the ether anesthesia. The heart is taken and stored immediately in a deep refrigerator (−80°C).

2.2. Enzyme-linked immunosorbent assay for HIF-1α and Mb

For quantification of HIF-1α and Mb proteins, enzyme-linked immunosorbent assay (ELISA) was used. Negative control was used in the ELISA protocol. Samples were prepared for ELISA by sandwich technique using Rat HIF-1α ELISA Kit-96T (Elabscience) for HIF-1α protein and Rat MYO ELISA Kit-96T (Elabscience) for Mb protein. The method was performed according to the manufacturer's instruction. First, mince the tissue to small pieces and rinse them in ice cold PBS (0.01 M, pH 7.4) to remove excess blood thoroughly. Then, one hundred milligram of rat heart tissues were homogenate in 1-mL PBS with a glass homogenizer on ice. To further break the cells, subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 min at 5000×*g* to get the supernatant. Tissue extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts of certain chemicals (Elabscience; 2014). An ELISA reader was used to measure the optical density at 450 nm in standards, blanks, and samples. Standard curve obtained with plotting the mean optical density value for each standard on the y-axis against the concentration on the x-axis and the equation based on the principle $y = ax + b$ was applied to determine concentration of HIF-1α and Mb proteins. Standard curve for HIF-1α protein is shown in Figure 2 and for Mb protein is shown in Figure 3.

2.3. RNA isolation and quantitative real-time reverse transcription polymerase chain reaction analysis for Mb

Total RNA was prepared from the rat heart tissue samples using Total RNA Mini Kit (tissue; Geneaid). The purity of the preparation was measured by the ratio between the absorbance at 260 and 280 nm using a spectrophotometer Varioskan Flash (Thermo Scientific). Primers of Mb and 18S as housekeeping genes were designed using online software from http://www.ncbi.nlm.nih.gov/. The primers of Mb and 18S are shown in Table.

In this study, real-time reverse transcription polymerase chain reaction was not used for HIF-1α expression. The HIF-1α messenger RNA (mRNA) translation into HIF-1α protein is highly efficient because it has an internal ribosome entry site. In the high mRNA

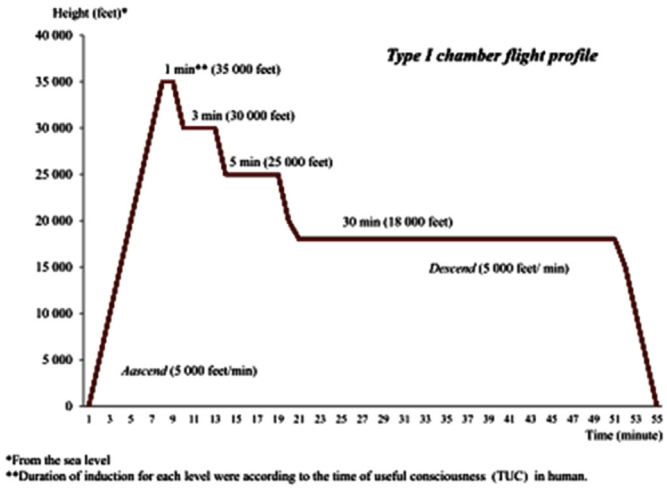

Figure 1. The simulation of flight plan of IHH exposure. Each procedure consists of 49.5 min of hypobaric hypoxia exposure. *From the sea level; **Duration of induction for each level was according to the time of useful consciousness (TUC) in human.

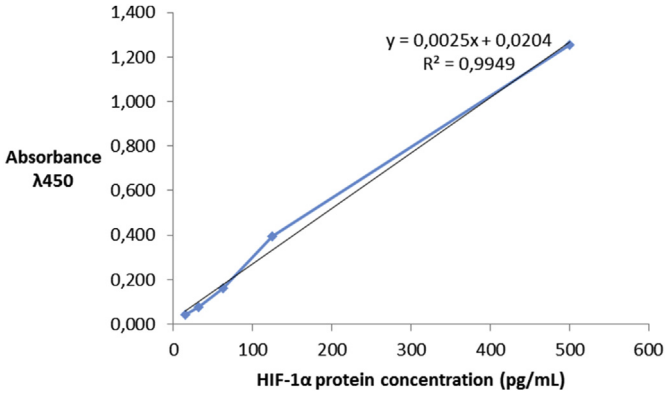

Figure 2. Standard curve for HIF-1α protein using the Rat HIF-1α ELISA Kit (Elabscience).

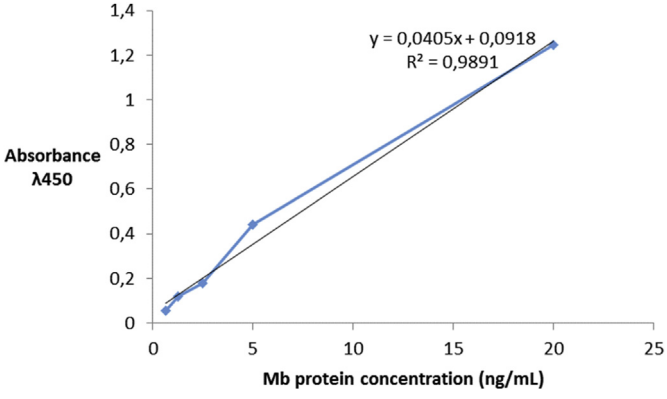

Figure 3. Standard curve for Mb protein using the Rat MYO ELISA Kit (Elabscience).

HIF-1α expression, not all mRNAs are translated into protein, which synthesis according to the need.

2.4. Statistical analysis

Statistical analysis was performed by using GraphPad Prism 5.0 (San Diego, CA). All data were expressed as mean ± standard deviation. Analysis of variance (ANOVA) for parametric test in normal and homogenous samples was used. If necessary, a nonparametric test was applied.

3. Result

3.1. Effects of IHH on HIF-1α protein level

HIF-1α protein was detected in the five groups by ELISA using anti-rat HIF-1α detection antibody (Elabscience), and the results are shown in Figure 4.

HIF-1α protein level directly increases in the HH group and gradually decreases in the IHH groups. However, HIF-1α protein levels in the IHH groups are lower than those in the control group (ANOVA, $p = 0.0437$).

3.2. Effects of IHH on Mb mRNA

Mb mRNA expression ratio in each group was calculated by the relative ratio of Mb to 18S, and the results are shown in Figure 5.

Mb mRNA expression ratio directly increases in HH group and gradually decreases in IHH groups. However, Mb mRNA expression ratio of IHH 3 × group is lower than that of the control group (ANOVA, $p = 0.0283$).

3.3. Effects of IHH on Mb protein level

Mb protein was detected in the five groups by ELISA using anti-rat Mb detection antibody (Elabscience), and the results are shown in Figure 6.

Mb protein level directly increases in HH group and gradually decreases in IHH groups. However, Mb protein level in IHH groups are lower than those in control group (ANOVA, $p = 0.0170$).

4. Discussion

4.1. Expression of HIF-1α in rat heart IHH

About 2 decades ago, Semenza and Wang *et al.* had found a protein factor which responded to hypoxia condition. The protein was called HIF, which in the following investigation was revealed as a transcription factor for EPO. HIF-1 is a transcription factor that regulates expression many genes. Many biological processes are regulated by HIF-1α–like erythropoiesis, angiogenesis, glucose metabolism, pH homeostasis, and blood pressure (Prijanti AR, 2010).

HIF-1α protein is always expressed by cells in the state of normoxia or hypoxia, but HIF-1α protein will experience a stabilization in a state hypoxia (Prabhakar *et al.*, 2012). Cai *et al.* showed that in heterozygous HIF-1α–deficient mice, the acute cardioprotection induced by ischemic preconditioning was absent, suggesting that HIF-1α is necessary for ischemic preconditioning (Cai *et al.*, 2003).

In this study, analysis of HIF-1α protein level in the rat heart, the results showed a significant difference between the control group and hypoxic rats (Figure 2). When compared with a control group

Table. Primer of Mb and 18S rRNA

Gene	Position (pb)	Sequences	Product of PCR (pb)	Temperature (°C)
Mb	201-222	F: 5′-TTCAGAGGACCTGAAGAAGCAC-3′	153	59.96
NM_021588.2	353-330	R: 5′-GAGATAAACTCCAGGTACTTGACC-3′		58.34
18S rRNA	881-900	F: 5′-CGCGGTTCTATTTTGTTGGT-3′	219	57.02
NR_046237.1	1099-1080	R: 5′-AGTCGGCATCGTTTATGGTC-3′		58.06

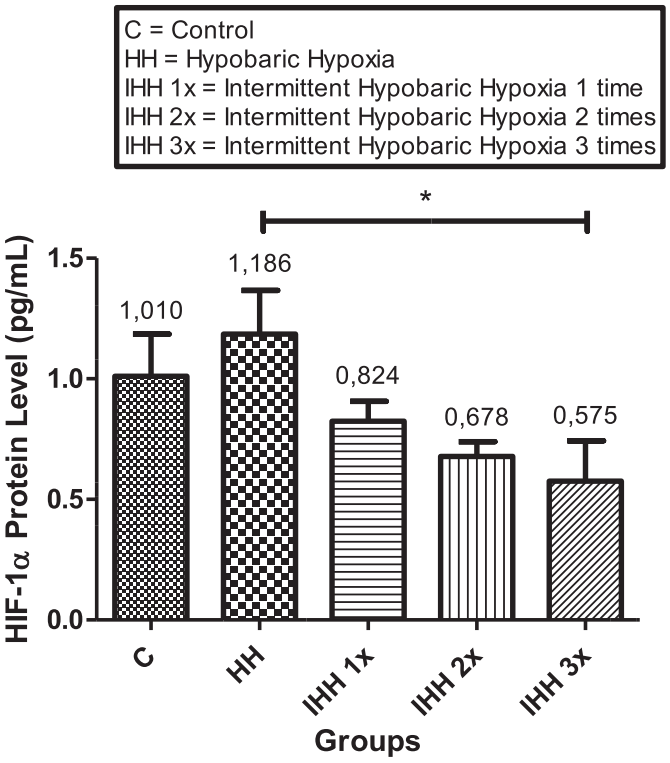

Figure 4. HIF-1α protein concentration in heart rat tissue after IHH exposure detected by ELISA using Rat HIF-1α ELISA Kit (Elabscience). *Significantly different than the control group ($p = 0.0437$, ANOVA test)

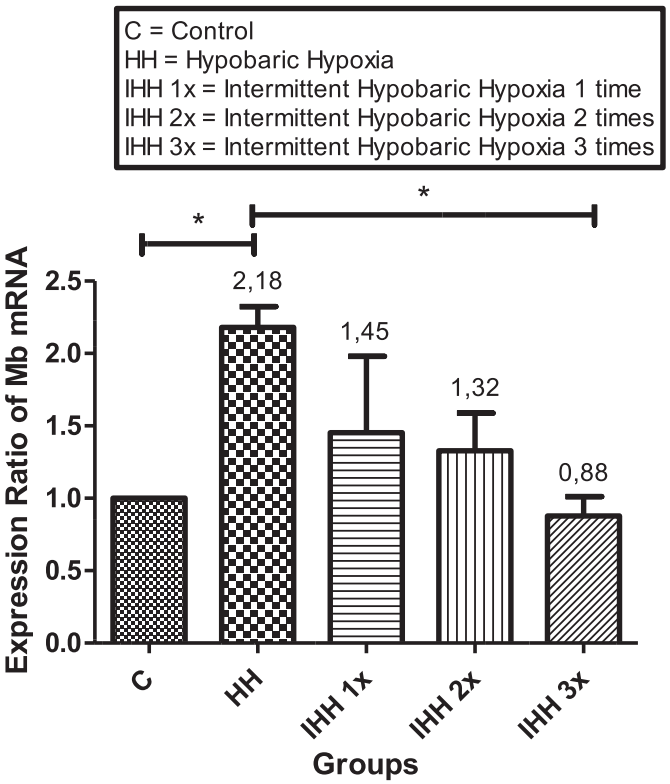

Figure 5. Mb mRNA expression ratio in heart rat tissue after IHH exposure. *Significantly different than the control group ($p = 0.0283$, ANOVA test)

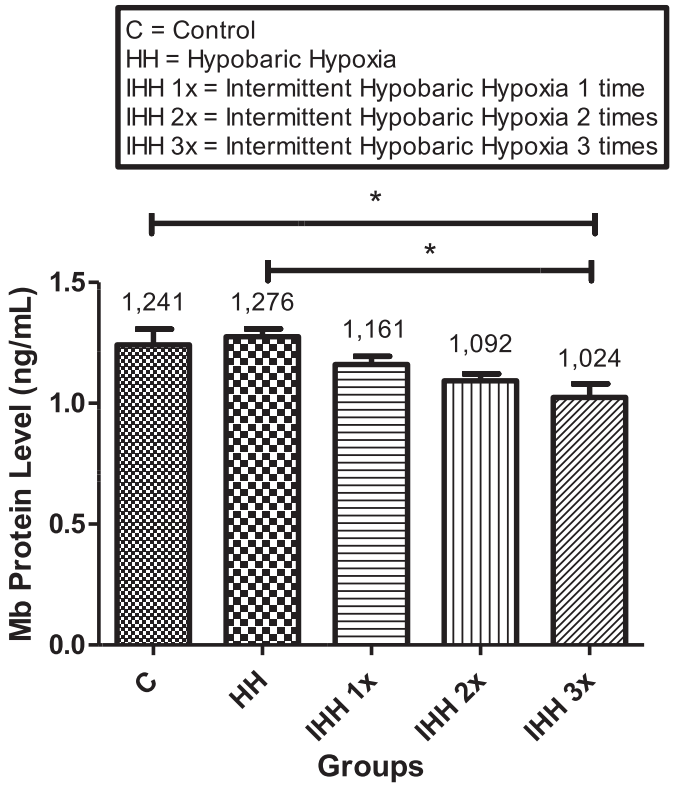

Figure 6. Mb protein concentration in heart rat tissue after IHH exposure detected by ELISA using Rat MYO ELISA Kit (Elabscience). *Significantly different than the control group ($p = 0.0170$, ANOVA test)

(C), HIF-1α protein level directly increases in HH group, then gradually decreases in IHH groups (IHH1 ×, IHH 2 ×, and IHH 3 ×). The highest level of HIF-1α protein is seen in the HH group. This means that there is an increase in HIF-1α protein level in hypoxic conditions and systemic adaptation occurs gradually at the cellular level in intermittent hypoxic conditions.

This increase is thought to occur because cells or tissues begin to adapt to hypoxic conditions. Liu states that the concentration of a protein involved in the mechanism of adaptation to hypoxia significantly increased, such as increased levels of HIF-1α protein that would regulate genes associated with angiogenesis, erythropoiesis, proliferation, differentiation, and apoptosis of cells (Liu *et al.*, 2007).

HIF-1α is a protein that is highly dependent on the oxygen content. The presence or absence of oxygen also regulates the levels of this protein. In normoxia conditions, when there is a sufficient amount of oxygen, HIF-1α will be degraded by the complex proteasome, whereas in hypoxic conditions, HIF-1α undergoes stabilization by forming a heterodimer with HIF-1β and then translocates to the nucleus and binds to the gene promoter HRE target (Prabhakar *et al.*, 2012).

4.2. Expression of Mb in rat heart IHH

Mb is a hemoprotein located in the cytoplasm. It functions as binding proteins and intracellular oxygen storage and facilitates the distribution of oxygen to the mitochondria in the process of energy formation (Amelia, 2011).

The yield on mRNA levels showed a significant difference between the control group and hypoxic rats (Figure 3). The yield on the protein level also showed a significant difference between the control group and hypoxic rats (Figure 4). When compared with the control group (C), Mb mRNA expression ratio and Mb protein level

directly increase in HH group then gradually decrease in IHH groups (IHH 1 ×, IIH 2 ×, and IHH 3 ×). The highest Mb mRNA expression ratio and Mb protein level were seen in the HH group. This means that there is an increase in Mb mRNA expression ratio and Mb protein level in hypoxic conditions, and systemic adaptation occurs gradually at the cellular level in intermittent hypoxic conditions.

The increased expression of Mb in the heart is closely related to the function of Mb in the regulation of blood flow during hypoxia through its potential as nitrate reductase enzyme, scavenger of nitric oxide (NO). At heart, its function as nitrate reductase is known to improve the viability of cardiac cells under ischemia conditions (Levett *et al.*, 2011).

Statistical analysis shows a very strong correlation between HIF-1α protein level and Mb mRNA expression ratio, which provides the prediction that regulation of the Mb expression is done by HIF-1α, which serves as a master regulator in oxygen homeostasis. Gilany mention that HIF-1α is also increasing the regulation of gene expression in iron metabolism, namely the formation of transferrin, and transferrin receptor plays an important role in the formation of the protein Mb (Gilany *et al.*, 2010). Mb is a protein required for binding and storage of oxygen that cells have a reserve of oxygen for metabolism.

In conclusion, measurement of HIF-1α protein level performed on heart tissue as a whole, without separating the parts. Overall results of this study indicate that the response of the genetic mechanisms of heart cells to the condition of a lack of O_2 is still running. This is evidenced by the increased protein level of HIF-1α in systemic hypoxic conditions, and adaptation mechanisms occur gradually on the conditions of intermittent hypoxia. This means that the protein HIF-1α is only synthesized as necessary, in accordance with the needs of the heart tissue.

Statistical analysis also showed a stronger correlation between the levels of HIF-1α protein with mRNA and protein Mb. Hintze and McClung mention that in hypoxia, the cell energy production decreased due to changes in the energy metabolism, i.e. when aerobic metabolism becomes anaerobic metabolism, but the absorption of iron (Fe) is increased to initiate the process of erythropoiesis and synthesis of proteins that require Fe as Mb (Hintze *et al.*, 2011).

Conflict of interest

The authors do not have any conflict of interest.

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