ABSTRACT

BACKGROUND: Intermittent hypobaric hypoxia is suggested to possess a protective effect toward the hypoxic condition. The aim of this study is to analyze the expression of cytoglobin (Cygb), neuroglobin (Ngb) and the specific activity of acetylcholinesterase (AChE) in brain tissue as adaptive responses to intermittent hypobaric hypoxia.

METHODS: Twenty-five adult Sprague-Dawley male rats were divided into 5 groups: 1) The control group (normoxia); 2) group exposed to acute hypobaric hypoxia (AHH); 3) group exposed to hypobaric hypoxia (HH) on day-1 and re-exposed on day-8 (intermittent hypobaric hypoxia once or IHH1x); 4) group that is exposed to HH on day-1, re-exposed to HH on day-8 and day-15 (intermittent hypobaric hypoxia two times or IHH2x); 5) group exposed to HH on day-1, re-exposed to HH on day-8, day-15 and day-22 (intermittent hypobaric hypoxia 3x or IHH3x). Homogenized brain tissue was then measured and analyzed for Cygb and Ngb protein expression, and also AChE specific activity.

RESULTS: Cytoglobin and Ngb were decreased in the acute induction and increased significantly along with the increasing frequency of the IHH induction. There were significant differences in Cygb expression between IHH2x and IHH3x groups compared to normoxia group, and between IHH1x, IHH2x and IHH3x compared to AHH group. There were significant differences in Ngb expression between IHH2x and IHH3x groups compared to normoxia group and between IHH2x and IHH3x groups compared to AHH group. The specific activity of AChE was increased significantly since the first induction of AHH, but then decreased in IHH3x. There were significant differences in the specific activity of AChE between IHH2x and IHH3x groups compared to normoxia and between IHH2x and IHH3x groups compared to IHH1x groups.

CONCLUSIONS: We conclude that IHH, especially IHH3x, seems to induce the protective adaptive response in the rat brain tissue through the changes of these three parameters.
Keywords
cytoglobin, neuroglobin, acetylcholinesterase, brain, intermittent hypobaric hypoxia

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**Introduction**

Cytoglobin (Cygb) and neuroglobin (Ngb) are globin proteins, found recently, which have special roles such as for oxygen supply in hypoxia. Both Cygb and Ngb have a high affinity for oxygen. These two globin proteins, which expressed by the neuron, are also known to act as scavengers of reactive oxygen species (ROS) and reactive nitrogen species. Increase in Cygb and Ngb occurs in hemorrhagic stroke patients with increased ROS. Therefore, it is suggested that an increase in Cygb and Ngb are adaptation responses to increase oxygen supply and scavengue ROS due to hypoxia in hemorrhagic stroke. Increased expression of Cygb also occurs after chronic systemic normobaric hypoxia induction in a rat's brain. An increased level of HIF-1α occurs along with the increased level of Cygb until day-7 of the induction, while the expression of Ngb was decreased in day 1, 3, 5, 7, and 14 of induction compared to normoxia.

Decreasing the level of oxygen causes energy production depleation needed by the cells for their optimal physiological function, such as a cognitive function of the brain. The cholinergic system is known to form synapses throughout the brain, including the cerebral cortex, which is part of the cognitive signaling pathway. Choliergic signaling is terminated, among other things, by the work of acetylcholinesterase (AChE), which catalyzes the hydrolysis of ACh in the synaptic cleft to an inactive form, choline and acetic acid.

In systemic chronic normobaric hypoxia, there was an increase of AChE specific activity, which proves that the induction is able to trigger an increase of the enzyme activity, so that it can reduce the cholinergic system function. In an animal study, bilateral common carotid artery occlusion caused chronic cerebral hypoperfusion. At week 4, the level of ACh in the hippocampus decreased. ACh reduction also occurred in the striatal area, and was suspected to be the cause of memory consolidation disturbance that leads to learning dysfunction.

Mulyawan’s research has shown that HIF-1α expression was increased after intermittent hypobaric hypoxia induction in a rat using hypobaric chamber. Until now, there is no data of how IHH affects Cygb and Ngb expression, which play a role in oxygen supply, and how this affects AChE specific activity, which plays a role in ACh, a neurotransmitter important for cognitive function, termination. The purpose of this study is to determine the effect of IHH toward these three parameters in the brain adult male of Sprague-Dawley rats.

**Methods**

**Animals**

All procedures were approved by the Ethics Committee of the Faculty of Medicine Universitas Indonesia Rumah Sakit Cipto Mangunkusumo No. 626/UN2.F1/ETIK/2014. All efforts were made to ameliorate any suffering of animals used in this research. The rats were observed daily to confirm their healthy condition. The behavior tests, done by Farhan[18], and the hypoxia induction did not involve painful stimuli.

Based on Federer’s rule, with five groups of induction, twenty-five adult Sprague-Dawley male rats from Badan Pengawasan Obat dan Makanan Republik Indonesia (BPOM RI), initially weighing 200–250 grams, were randomly divided into 5 groups: 1) The control group (normoxia); 2) group exposed to acute hypobaric hypoxia (AHH, control to intermittent hypobaric hypoxia (HH) treatment); 3) group exposed to HH on day-1 and re-exposed on day-8 (intermittent hypobaric hypoxia once, IHH1x); 4) group exposed to HH on day-1, re-exposed to HH on day-8 and day-15 (intermittent hypobaric hypoxia 2 times, IHH2x); 5) group exposed to HH on day-1, re-exposed to HH on day-8, day-15 and day-22 (intermittent hypobaric hypoxia three times, IHH3x).

Before the experiment all rats were kept under optimal conditions: 12:12 hours light to dark cycle at 24°C, in the animal house of Indonesian Air Force Institute of Aviation Medicine, LAKE-SPRA dr. Saryanto, Jakarta, with food and water ad libitum. Pelleted food and tap water were given. Wire cages were used to ensure optimum ventilation; subjects in a group were put in the same cage. Shredded newspaper were used and changed daily.

After treatment, rats were euthanized by cervical dislocation. Then the brains were immediately removed, weighed and divided into aliquots and stored in -80°C.

The procedure of hypobaric hypoxia is referred to hypobaric hypoxia type I chamber flight profile, modified by Mulyawan, presented in Figure 1.

In this procedure, starting around 09.00 AM, the rats were put in a hypobaric chamber at the Aerophysiology Department of Indonesian Air Force Institute, LAKE-SPRA dr. Saryanto, Jakarta, and exposed to treatment ascending with rising speed of 5,000 feet/minute until reach the altitude which equal to 35,000 feet above sea level for 1 minute, then descending gradually to 30,000; 25,000; 18,000 feet for 3; 5; and 30 minutes respectively. And then descend gradually until reaching the sea level. All descending speed is performed at 5,000 feet/minute.

**Sample preparation**

Brain tissue was homogenized using RIPA Lysis Buffer (Santa Cruz®, product number sc-24948) before the measurement of the total protein, Cygb and Ngb proteins, using ELISA method and specific activity of AChE. These activities were conducted at the Laboratory of Molecular Biology for Oxidative Stress Studies, Department of Biochemistry and Molecular Biology, Faculty of Medicine Universitas Indonesia, Salemba, Jakarta.
Total protein concentration of brain tissue
Total protein concentration is determined using bovine serum albumin (BSA) as standard solution (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 mg/dL) and absorbance was read at $\lambda$ 280 nm using Nano drop Varioskan Flash (Thermo Scientific).

Cytoglobin concentration of brain tissue (Rat Cytoglobin ELISA Kit USCN ®, product number SEC426Ra)
Cygb protein concentration was measured using ELISA method. The microplate provided in the kit has been pre-coated with Cygb specific antibody. The standard solutions of Cygb were made as follows: 0 (blank); 0.78; 1.56; 3.12; 6.25; 12.5; 25; and 50 ng/mL. The absorbance was read at $\lambda$ 450 nm. The concentration of Cygb was measured using Cygb standard curve. The concentration of Cygb in brain tissue was presented as the concentration of Cygb/total brain protein concentration (ng/mg protein).

Neuroglobin concentration of brain tissue (Rat Neuroglobin ELISA Kit Cusabio ®, product number CSB-E08738r)
Ngb protein concentration is determined using ELISA method. Ngb specific antibody has been pre-coated onto the microplate. The standard solutions of Ngb were made as follows: 0 (blank); 15.6, 31.2, 62.5, 125, 250, 500, and 1000 ng/dL. The absorbance was read at $\lambda$ 450 nm. The concentration of Ngb was measured using Ngb standard curve. The concentration of Ngb in brain tissue was presented as the concentration of Ngb/total brain protein concentration (ng/mg protein).

Acetylcholine specific activity assay (Sigma-Aldrich ®)
In this assay, thiocholine as the product of AChE is reacted to 5,5'-dithiobis (2-nitrobenzoic acid, DTNB) to form a yellowish color product, which provides absorption at $\lambda$ 412 nm. The intensity of the yellowish color is equivalent to the activity of AChE. One unit of AChE is a number of enzymes that catalyze the production of 1.0 µmol tiocholine/minute at pH 7.5 at room temperature. The specific activity of AChE was measured by dividing the AChE activity with the total protein of each sample (U/mg protein).

Statistical analysis
Graph Pad Prism version 7.0 was used for statistical analysis. Data normality was examined using D’Agostino and Pearson normality test. Then data were analyzed using ANOVA for significance and further post hoc with Tukey test. The Pearson correlation test was used to examine the correlation between parameters.

Results
Cytoglobin concentration in brain tissue during IHH1x, IHH2x, and IHH3x compared to AHH and normoxia
The concentration of Cygb in brain tissue after induction of AHH, IHH1x, IHH2x, IHH3x, and normoxic groups, is shown in Figure-2. In this figure, we can see that there were significant differences between the IHH2x and IHH3x groups compared to normoxic group (p=0.015 and p=0.000, respectively). There was also a significant difference between IHH2x and IHH3x groups compared to the AHH group (p = 0.005 and p=0.000, respectively). Differences also appeared between IHH1x and IHH3x (p = 0.005).

Neuroglobin concentration in brain tissue during IHH1x, IHH2x and IHH3x compared to AHH and normoxia
The concentration of Cygb in brain tissue after induction of AHH, IHH1x, IHH2x, and IHH3x, and normoxic groups, is shown in Figure-2. In this figure, we can see that there were significant differences between the IHH2x and IHH3x groups compared to normoxic group (p=0.015 and p=0.000, respectively). There was also a significant difference between IHH1x, IHH2x and IHH3x compared to the AHH group (p=0.02, p=0.001 and p=0.000, respectively). Differences also appeared between IHH1x and IHH3x (p = 0.005).

Neuroglobin concentration in brain tissue during IHH1x, IHH2x and IHH3x compared to AHH and normoxia
It is shown in Figure-3 that there are significant differences between the IHH2x and IHH3x groups compared to normoxic group (p=0.049 and p=0.03, respectively). There were also significant differences between IHH2x and IHH3x groups compared to the AHH group (p = 0.005 and p=0.000, respectively).
Differences were also showed between IHH2x and IHH3x groups compared to IHH1x group (p = 0.022 and p=0.001, respectively).

Specific activity of AChE in brain tissue of rat exposed to normoxia and AHH
As can be seen in Figure 4, there were differences in the specific activity of AChE in brain tissue of rats in IHH2x and IHH3x groups compared to normoxic group (p=0.000 and p=0.000, respectively). There were also significant differences between IHH2x and IHH3x with the AHH group (p=0.000 and p=0.000, respectively). There were also significant differences between the IHH2x and IHH3x groups compared to IHH1x group (p=0.000 and p=0.000, respectively), and between IHH2x and IHH3x (p=0.026).

Correlation between Cygb protein concentration and specific activity of AChE
There was a strong positive correlation between the Cygb protein and specific activity of AChE in brain tissue of normoxic, AHH and IHH groups (Pearson, r = 0.728 and p<0.0001) (Figure-5a).

Correlation between Ngb protein concentration and specific activity of AChE
There was a moderate positive correlation between the Ngb protein and specific activity of AChE in brain tissue of normoxic, AHH and IHH groups (Pearson, r = 0.542 and p<0.0001) (Figure-5b).
Comparison of expression patterns between Cygb, Ngb levels, and AChE specific activity

Comparison of the expression patterns between Cygb, Ngb levels and the specific activity of AChE is shown in Figure-5c. It appears that Cygb and Ngb expressions were decreased while the specific activity of AChE was increased at AHH. At the induction of IHH1x, Cygb and Ngb expressions were increased, so did the specific activity of AChE. Induction of IHH2x caused an increase in Ngb and Cygb levels and also the specific activity of AChE. At the induction of IHH3x, Cygb and Ngb expressions were increased, however, the specific activity of AChE was decreased.
Discussion

In this research, Cygb and Ngb expressions of AHH group were decreased compared to normoxia. Those two protein expressions were then increased in intermittent induction of hypoxia, from IHH1x induction until IHH3x. The low expressions of Cygb and Ngb in AHH group might be due to the role of these proteins in scavenging ROS produced in this induction. In repeated hypobaric hypoxia induction (IHH1x, IHH2x, and IHH3x), the increase of Cygb and Ngb expressions might be due to adaptation response toward hypoxia and the increased ROS in this condition. Cygb and Ngb are known to be increased in supplying oxygen and reducing or scavenging ROS in hypoxic conditions.

The increase of specific activity of AChE was seen from AHH induction and continued to increase until IHH2x and then decreased in IHH3x. The increased specific activity of this enzyme was also reported by Muthuraju et al. in hypobaric hypoxia and Andriani et al. in chronic normobaric hypoxia. An increase in AChE activity, which then caused the decrease of ACh concentration, in and around amyloid ß-peptide (Aβ) (a substance which found to be increased in Alzheimer’s disease) has also been shown. The increased activity of AChE which causes the decrease amount of ACh eventually caused a reduction in blood flow. The reduction of blood flow due to the decreased levels of ACh is due to the function of ACh in causing vasodilatation. In brain hypoperfusion by ligating common carotid artery, there is a decrease in ACh in the hippocampal area, which also related to memory and learning impairment. In addition, the loss of perivascular cholinergic terminals was shown in AD patients compared to aged controls. This research was supported by other researchers who observed impaired cortical blood flow in patients with AD.

Moreover, AChE inhibitor medication is known to affect cholinergic function in subjects treated with hypobaric hypoxia (decrease acetylcholinesterase activity, increase acetylcholine levels and upregulation of choline acetyltransferase—an enzyme that has a role in acetylcholine formation) and eventually memory function. Inhibitors of AChE are also known to improve cerebrovascular function. This medication is known to overcome the cognitive function impairment in Alzheimer’s disease. As a comparison in cognitive function, Farhan reports that IHH, using the same procedure with our study, increases cognitive function compared to control group.

In this research, the specific activity of AChE was increased in AHH1x conditions, which meant that there was a decrease in blood flow to the brain due to the decrease of ACh. This condition may lead to a decrease in blood flow, inducing the adaptation response through increased expressions of Cygb and Ngb since IHH1x until IHH3x to increase blood/oxygen supply. However, since the blood flow was not measured in this research, this limitation needs further research to ensure whether this hypothesis is correct. Meanwhile, in IHH3x there was a decrease in AChE specific activity. It suggests that in IHH3x the blood/oxygen flow is sufficient due to the increase expressions of Cygb and Ngb which cause an increase in oxygen availability. This phenomenon is supported by Mulyawan’s research that reports an increase in microvascular density using GLUT-1 as the marker from IHH1x until IHH3x induction. However, the mechanism of the increase of AChE activity in hypoxia requires further study.

The present research has demonstrated that intermittent hypoxia (IHH1x until IHH3x) can provide protection against hypoxia, which was shown in the increase of Cygb and Ngb that supply oxygen. Overall, this research could be implemented for individual training to adapt to hypoxia conditions, for example, air force pilot.

Conclusions

We conclude that IHH seems to induce a protective adaptive response in the rat brain tissue through the changes of Cygb and Ngb expression and the changes of AChE specific activity. Further research is needed to measure and evaluate the blood flow changes and the ACh level and choline-acetyltransferase (ChAT), an enzyme that catalyzes ACh synthesis, using the same research model.

Data availability

Dataset 1: Raw data for cytoglobin, neuroglobin and acetylcholinesterase specific activity measurements 10.5256/f1000research.13592.d197006

Competing interests

No competing interests were disclosed.

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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References


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The paper by Masengi et al. reports that periodic hypobaric hypoxia up-regulates cytoglobin and neuroglobin levels and affects AChE activity in the brain. The study is potentially interesting but needs extensive revision to reach the level of acceptance.

Major criticism:

1. The model used cannot be referred to as an "intermittent (hypobaric) hypoxia". In fact this is periodic (or repetitive) hypoxia. Intermittent hypoxia is a mode when lasting hypoxia is interrupted by brief episodes of normoxia, and this phenomenon is widely studied worldwide.

2. The mode of ascending the altitude looks puzzling: very fast elevation to a height about 10 km (speed is about 1.5 km/min that can itself produce many side-effects), and strange dynamics of the whole procedure. The feasibility of such model must be proved and explained to the reader. The authors refer to unpublished PhD thesis of Mulyawan (2012), as well as to the poster at The 1st Asian Researcher Symposium at the Universitas Indonesia-Depok, that cannot be accepted as reliable justification of the model.

3. Neither the negative effect of single exposure nor the protective action of the repetitive exposures was demonstrated or even convincingly cited. The only reference to unpublished PhD thesis of Farhan (2016) reporting some cognitive improvement is not enough.

4. Interpretation of the results needs much attention, e.g. no statistic differences with control are seen following AHH for Cygb and Ngb but authors write: "In this research, Cygb and Ngb expressions of AHH group were decreased compared to normoxia". Similarly, no differences in Cygb and Ngb expression where detected between IHH2x and IHH3x, therefore it cannot be interpreted as "especially IHH3x", etc.

5. Methods section, page 3, “After treatment…” - It is unclear at what time after hypoxic exposures the tissue samples were obtained. In case if the samples were taken immediately after hypoxia (that is 1 h from the onset of hypoxic trial) it is possible that it is not enough time to see the changes at the level of proteins. This might account for no changes revealed after single HH episodes (termed AHH). In this case it is a methodological failure.

Minor questions:
1. Fig. 5 is redundant; it litters the article and should be removed. It adds no information.

2. Some concern arises regarding the data on AChE activity and their interpretation. It is incorrect to conclude on the changes in cholinergic system based only on the parameter examined. We would recommend to additionally examine the AChE localization and its isoform composition. A rate of synthesis (ChAT activity) vs. degradation (AChE activity) processes should be also kept in mind. In addition, AChE performs actions other than degradation of Ach, e.g. is involved in cellular adhesion processes which play roles in neuroplasticity and neuroprotection.

3. The language style and grammar should be thoroughly edited.

Is the work clearly and accurately presented and does it cite the current literature?  
Partly

Is the study design appropriate and is the work technically sound?  
Partly

Are sufficient details of methods and analysis provided to allow replication by others?  
Yes

If applicable, is the statistical analysis and its interpretation appropriate?  
Yes

Are all the source data underlying the results available to ensure full reproducibility?  
No source data required

Are the conclusions drawn adequately supported by the results?  
No

Competing Interests: No competing interests were disclosed.

Referee Expertise: Hypoxia/ischemia, hypobaric hypoxia, brain, neuroprotection, cerebral mechanisms of hypoxic tolerance, expression of genes and proteins, neurochemistry

We have read this submission. We believe that we have an appropriate level of expertise to state that we do not consider it to be of an acceptable scientific standard, for reasons outlined above.

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