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PREFACE

As a scientific organization that accommodates scientific activities in the field of its member's expertise, a scientific journal is an absolute necessity. **PBBMI (formerly known as PERHIBI)** already has its own journal entitled ***Acta Biochimica Indonesiana*** which was first published in 1992. The effort to publish journals regularly is hard work, which requires high dedication for the organizers, and faces various obstacles, especially regarding the source of writing to be published. Even though Acta Biochimica Indonesiana was published several years with a frequency of 2 times a year by the struggle of the previous PERHIBI management, but since 2002 this journal has not been published for various reasons.

During the 2015-2018 PBBMI management period, with a rethinking of the purpose of the organization, namely to provide a forum for all scientific activities to advance and develop knowledge in the field of biochemistry and molecular biology as wide as possible, this journal was revived with the hard work of the management. With a truly sincere effort, finally, the Journal can be published in 2 times volume a year in June and December.

Hopefully, the presence of the PBBMI journal, ***Acta Biochimica Indonesiana***, will trigger the desire of all members to write and submit their research to be communicated here, and at the end, will stimulate Indonesian researchers to be better known in the international arena.

President of ISBMB

Rahmawati Ridwan, Apt, MS, PhD

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CONTENT

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ACTA BIOCHIMICA INDONESIANA

INSTRUCTION FOR AUTHOR

Manuscripts should be written and published in English. Authors should refer to the guideline below when preparing their manuscripts. Previously published manuscripts (either in whole or in part) will not be considered.

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1.1. Research article

Research article are original and innovative reports which represent the major advance in biochemistry or molecular biology with deep discussion in the understanding of a significant problem. They should have no more than 50 references. Research article usually typed between 3,000 ~ 5,000 words of text (including references, note and captions) are expected to present a major advance. Experimental procedure should be given in sufficient detail to support the reports.

1.2. Short communication

Short communication are short report that presenting preliminary result with potentially exceptional relevance or giving details of new models, innovative methods, techniques or apparatus. Short communication article should be no more than 2500 words (including references, notes and captions) and must at least has 10 primary references.

1.3. Review article

Review article are summaries of excellent scientific research articles which deal with specific research area of high interest in any area that fall under biochemistry and molecular biology scope. Review article can be written up to 5000 words (including references, note and caption) and around 80% of references must be come from primary articles.

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Manuscript should be written clear and simple so that they are accessible to readers in other disciplines. The decision of the publication will be notified to the authors using email or phone. Repeated submission of the same paper will not be acknowledged. All paper submission will be treated as confidential documents. Our peer review process is also confidential and the identities of reviewers are not released. Editor will decide who will review the article and it is not necessary to include reviewer provided by the author. Research paper that are selected for in-depth review are evaluated by at least two outside reviewers. Reviewers are contacted before we send the documents and asked to return comments within 1 to 2 weeks. Selected papers are edited to improve accuracy and clarity and for length.

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Manuscript should be between 9 – 15 pages (including tables and figures).We only receive digital file of the manuscript (word file), authors must ensure that the PC used to write the manuscript is free of virus. All manuscript (research article and short communication) should be divided into the following headings:

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- Author's information
- Abstract
- Keywords
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- Methodology
- Results
- Discussion
- Acknowledgement
- References
- Tables
- Figures

Format for review article is the same as research article except "materials and methods" and "results" were replaced with "Review".

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The title page should include complete manuscript title (no more than 25 words) and a short title which will be used as a "running head". Short title should not exceed 60 characters including space.

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State the objectives of the study and provide an adequate background, avoiding a detailed literature survey or to describe the results.

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Methods should include relevant details on the materials and experimental design and techniques so that the experiments can be repeated. Names of products and manufacturers should be included only if alternate sources are deemed unsatisfactory. Novel experimental should be described in detail. If previous researcher method is used, please describe briefly that method and describe in detail if you make any modification to the procedure.

3.7. Results

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This should directly relate to the results of the study. Do not provide a general review of the topic.

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You may state the main conclusion of your study. If possible, provide recommendations or suggestion for further research.

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Acknowledgements should be made only to those who have made a substantial contribution to the study.

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article(s) but not yet published should be sent as manuscript appendix. Website, personal communications, unpublished data should be incorporated in the text in parentheses. Example: ... to search the similar gene with obtained DNA sequence, comparation for homology was done by BLAST search (http://www.ncbi.nlm.nih.gov/blast). Personal communication need to be written with the initials surname of the source accompanied with the year of the statement, e.g., (William 2007, personal communication). Authors should provide proof(s) of the communication. Please follow in detail all examples below

Article:
Hallal AH, Amortegui JD, Jeroukhimov IM, Casillas J, Schulman CI, Manning RJ, et al. Magnetic resonance cholangiopancreatography accurately detects common bile duct stones in resolving gallstone pancreatitis. J Am Coll Surg. 2005; 200(6) :869-75.

Book:
Murray PR, Rosenthal KS, Kobayashi GS, Pfaller MA. Medical microbiology. 4th ed. St. Louis: Mosby; 2002. Chapter in a book: Rosenberg GA. Matrix metalloproteinase and proteolytic opening of the blood brain-barrier in neuroinflamation. In: deVries E, Prat A, editors. The Blood-brain Barrier and Its Microenvironment Basic Physiology To Neurological Disease. New York: Taylor and Francis Group; 2005. p.335-58.

Dissertation/ Thesis/ Essay:
Arlauckas SP. Near infrared fluorescent choline kinase alpha inhibitors for cancer imaging and therapy [Dissertation]. Philadelphia: University of Pennsylvania; 2015.

Part of website/monograph:
Medline Plus [Internet]. Bethesda: US National Library of Medicine; ©2009. Diabetic Kidney Problems [update 2015 Nov 2; cited 2015 Nov 16]. Available from: https://www.nlm.nih.gov/medlineplus/diabetickidneyproblems.html/.

Conference Paper:
Fledelius HS. Myopia and significant visual impairment: global aspects. In: Lin LLK, Shin YF, Hung PT, editors. Myopia Updates II: Proceedings of the 7th International Conference on Myopia 1998 Nov 17-20, Taipei. Tokyo: Springer; 2000. p.3-17.

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3.13. Figures
Submit each figure as a separate file and in TIFF or EPS format with a resolution of at least 300 dpi. Number figures consecutively using Arabic numerals. Submit photographs scaled as near to printed size as possible. Arrows should be given to point certain objects. If magnification is significant, indicate using a bar on the print rather than a magnification factor in the figure legend (see "**10. Example**"). Give each figure a legend containing sufficient information to make the figure intelligible without the reader having to refer to the text. Key all the legends together. If a figure has been published previously, acknowledge the original source and submit written permission from the copyright holder to reproduce it.

3.14. Statistical graphs
Give standard deviation to every mean value. Authors that used Microsoft Excell Program need to give the raw data.

4. Abbreviations and Units
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is important to maintain the capital letters and lower case letters as they appear in the abbreviation to avoid confusion with other abbreviations. In devising such abbreviations and symbols, the recommendations of the Nomenclature Committee of IUBMB and the IUPAC-IUBMB Joint Commission on Biochemical Nomenclature (see http://www.chem.qmul.ac.uk/iubmb/) should be followed as far as practicable.

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During manuscript submission, the submitting author must provide contact information (full name, email address, institutional affiliation and mailing address) for all of the co-authors. The author who submits the manuscript for publication accepts the responsibility of notifying all co-authors that the manuscript is being submitted. Deletion of an author after the manuscript has been submitted requires a confirming letter to the Editor-in-Chief from the author whose name is being deleted.

Upon submission, Editor in Chief will assign the manuscript to a corresponding Editor for further handling. The Editor will request at least two scientists to review the manuscript. Based on the comments from the reviewers, Editor, and Editor-in-Chief will make the decision on the manuscript.

Authors should review the preparation of the manuscripts prior to submission of a manuscript. Close attention to all the required details in preparation of the manuscripts will expedite review and reduce the time to publication.

9. Journal Publishing Agreement

A properly completed and signed Journal Publishing Agreement must be submitted for each manuscript. Authors must signed the agreement stating that the submitted article have never been published on another Journal.

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Figure template using scale bar

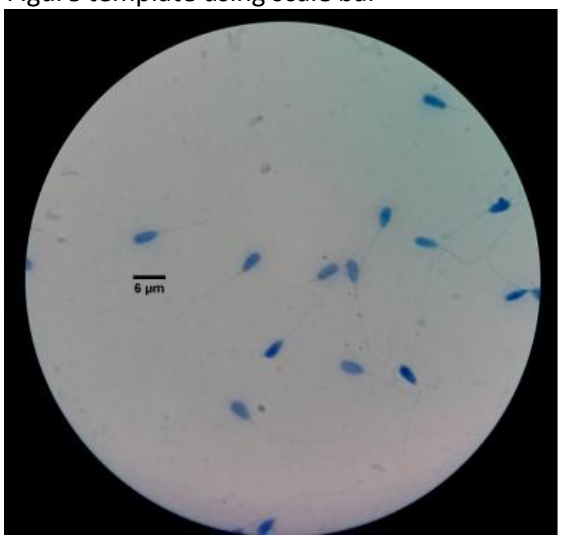

Figure 1. Sperm assay using *Halomax* kit to detect DNA fragmentation

Table 2. BLASTN result of Rat Transferrin mRNA sequences

Max Score	Description	Accession
475	Rat transferrin mRNA, partial cds	M26113.1
315	Rattus norvegicus Cc1-8 mRNA, complete cds	AY325241.1
254	Mus musculus transferrin mRNA, complete cds	AF440692.1

ACTA BIOCHIMICA INDONESIANA

RESEARCH ARTICLE

ENHANCING COGNITIVE FUNCTION OF HEALTHY WISTAR RATS WITH AQUEOUS EXTRACT OF *Centella asiatica*

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ABSTRACT

Background: *Centella asiatica* (L.) Urb is a native herb from Asian countries such as India, China, and Indonesia. This herb has been widely used as a cure for various diseases. However, studies investigating the aqueous extract of *Centella asiatica* as a nootropic in healthy individuals are still very limited.

Objective: This study aims to investigate the potential of aqueous extract of *Centella asiatica* in enhancing cognitive function of healthy male Wistar rats.

Methods: Rats were randomly allocated to four treatment groups, i.e. without treatment and aqueous *Centella asiatica* extract at doses of 200, 400 and 800 mg/kg. To determine enhancement of cognitive function, novel object recognition (NOR) test was conducted after the course of treatment. Acetylcholine content was assessed by enzyme-linked immunosorbent assay.

Results: There was a significantly high preference index towards the novel object in the NOR test in groups treated with 200 mg/kg and 800 mg/kg of the aqueous extract compared to control. This was further confirmed by a significant increase of brain acetylcholine content in rats treated with 200 mg/kg of the extract.

Conclusion: Therefore, this study confirms that the aqueous extract is effective in enhancing cognitive performance of healthy Wistar rats.

Keywords : *Centella asiatica*, Acetylcholine, Novel object recognition, Cognitive function, Cognitive performance

Received Nov 26, 2018 ; Revised 20 Dec, 2018 ; Accepted 26 Dec, 2018

INTRODUCTION

Centella asiatica L, (Urb) also known as pegagan (Indonesia) or gotu kola (India), is a plant from the family of Apiaceae present in several Asian countries.[1] This plant has long been used to cure a number of conditions in traditional Indian medicine, such as skin eczema, leprosy, diarrhea, amenorrhea and dementia.[2] In the last two decades, there have been studies reporting the effect of *Centella asiatica* (CA) extract on various diseases, particularly in the field of neuroscience. Both whole extract and isolated compounds of CA have been shown to possess antioxidant effects and are able to modulate brain neurotransmitters thereby contributing to the improvement of epilepsy [3], anxiety [4], and cognitive function deficits.[5,6]

Centella asiatica extract has been shown to facilitate increased acetylcholine levels in the brain.[7] Acetylcholine is an ester product of acetic acid and choline, secreted by both central and peripheral nerve cells. It plays a role in improving memory and learning through its activity on nicotinic receptors.[8] The activation of these nicotinic receptors has been shown to modulate the course of Alzheimer's disease, resulting in improved cognitive function.[9] Thus, CA extract is a potential drug for individuals with cognitive deficit due to the course of a disease. However, studies investigating the potential of CA extract as a supplement in healthy individuals, are very limited. This supplemental use is based on the concept of nootropics in enhancing cognition in healthy individuals. Cognitive enhancers are generally used to alleviate cognitive deterioration in individuals with neurological diseases.[10] However, research has shown that even in 'healthy' subjects (without neurological complaints), a decrease in cognitive performance is also possible, which can be due to oxidative stress.[11,12] This provides a plausible rationale for the use of cognitive enhancers in healthy subjects. Furthermore, Furey et al.[13] confirmed that healthy individuals treated with the cholinesterase inhibitor, physostigmine, exhibited enhanced cognition. Therefore, this study aimed to investigate the properties of aqueous CA extract in enhancing cognitive function of healthy Wistar rats.

MATERIAL AND METHODS

The present study was conducted at the Biotechnology and Animal Laboratory at the Faculty of Medicine, Universitas Sriwijaya, Palembang, Indonesia. This study was reviewed and ethically approved by the Commission of Ethics in Medical Research of the Faculty of Medicine, Universitas Sriwijaya (Ethical Approval Certificate No. 130/kepkrsmhfkunsri/2017). Experiments related to the use of animals has complied with the relevant regulations for the care and use of animals. General experimental procedures in this study are depicted in Figure 1.

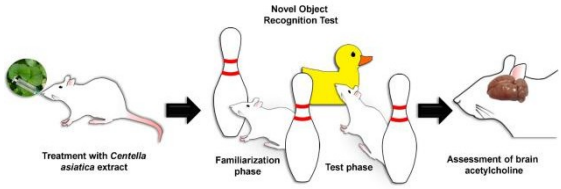

Figure 1. Experimental procedures

Animals

This study used 24 healthy white male Wistar rats, aged 2-3 months, weighing 200-300 g. Rats were obtained from a registered farm in Bandung, Indonesia. Rats were examined by a registered veterinarian and confirmed with

a health certificate issued by the local Livestock Division. Rats were housed at a temperature of 25±2°C and 12 hours light:dark cycle with food and water available *ad libitum*. They were acclimatized for 7 days before being randomly allocated into four experimental groups, i.e. control (untreated), and aqueous *Centella asiatica* extract at doses 200 mg/kg, 400 mg/kg, and 800 mg/kg.

Plant Extract

The plant was verified as *Centella asiatica* (L.) Urb (local name *pegagan* or *kaki kuda*) by the Plant Taxonomy Laboratory of Universitas Sriwijaya. as issued in the Letter of Plant Determination No. 174/UN9.1.7/4/PP/2017.

The extract was freshly prepared from aerial parts of CA. Plants were cultivated and air-dried for 10 days. Dry aerial parts were ground until fine powder was formed. The powder was weighed and brewed in distilled water which was already heated at 90°C with a ratio of 1:20 (1 gram of powder in 20 ml of distilled water). The mixture was allowed to stand for 30 minutes at room temperature before undergoing filtration. This was repeated twice to get a maximum yield of extract from the same batch of powder. The filtrate was then evaporated in a rotary evaporator (Heidolph, Germany) according to the manufacturer's settings for aqueous solvent (vacuum 72 mbar, rotation 50 rpm, vapor temperature 32.1°C, and bath temperature 60°C) until the extract volume decreased to 10-15% initial volume before being dried further. The dried extract was weighed accordingly and re-suspended in distilled water for experimental use. Three doses were used in this study, 200 mg/kg, 400 mg/kg, and 800 mg/kg. The extract was given per oral by using an oral-gastric tube with a final volume of 2 ml.

Novel Object Recognition Test

After acclimatization, rats were given treatment accordingly for 21 days before being evaluated by the novel object recognition (NOR) test. NOR test is a behavioral test that can be used to detect the efficacy of a drug on cognitive function.[14] This test is based on the internal drive of rats to explore unfamiliar objects (novel objects) in their surroundings. According to Ennaceur and Delacour[14], the average rat spends about 15 minutes to 1 hour to explore new objects in its surroundings.

This study used a modified protocol by Mathiasen et al.[15] The test was performed within one day, aiming for a short retention interval, which is 15 minutes. Rats were placed into a 50 x 50 x 35 cm plastic box with opaque walls lined with black cardboard on the outer surface. A camera was mounted on top of the box to record rat behavior during the test. The test was divided into 3 phases, the habituation phase (3 minutes), the familiarization phase (3 minutes), and the test phase (3 minutes).

In the habituation phase, rats were placed inside the empty box for 3 minutes. In the familiarization phase, the box was filled with 2 identical objects in the form of the same colored plastic bowling pins which were oppositely arranged in a diagonal fashion. Rats were placed in the middle of both objects, facing the wall of the box to avoid bias preference against one of the objects. They were left to explore both objects in the box for 3 minutes. After that, rats were returned to their cage for 15 minutes for memory retention.

In the 3-minute test phase, one of the bowling pins was replaced with a novel object in the form of a plastic duck. The

researcher recorded and calculated the time required for the rats to explore both the familiar (bowling pin) and the novel object (duck). The criteria of rat exploration was if the animal directed its nose towards the object within a distance of ≤ 2 cm and/or stuck its nose to the object. The distance was confirmed by comparing the experimental videos to a control video where the distance was measured real-time. The duration of exploration on the familiar object (bowling pin) and novel object (duck) was calculated as the preference index, which was expressed as the percentage of the exploration time for the familiar or novel object against the total exploration time for both objects.[16]

Enzyme-linked Immunosorbent Assay (ELISA)

After the 21-day treatment, rats were sacrificed by inhalation of chloroform, and whole brains were evacuated. Specimens were weighed before being minced with scissors. To inhibit the activity of acetylcholinesterase on the tissues, tubes containing the specimens were placed into boiling water for 10 minutes.[3] This study used an ELISA kit (Sunlong, China) for rat acetylcholine in accordance to the manufacturer's instructions. The sample was further homogenized with a tissue homogenizer, then centrifuged at 867 g for 20 minutes at 4°C. The supernatant was transferred into a new tube for ELISA. This study used 6 concentrations of the acetylcholine standard (Sunlong, China) for the determination of the standard curve. The ELISA plate was inserted into the microplate reader to determine absorbance (opticdensity-OD) at a wavelength of 450 nm.

Phytochemical screening

Phytochemical screening was performed to identify the class of active compounds contained in the aqueous extract. Identification of alkaloids was carried out using Wagner's test, whereas steroid/triterpenoids used Lieberman-Burchard method.[17] Tannin and phenol were assessed by addition of 1% and 2.5% $FeCl_3$, respectively. Saponin was evaluated by addition of distilled water followed by vigorous shaking. Flavonoid was identified by using thin layer chromatography (TLC) using an F254 silica gel plate (Merck, Germany) developed with 60% ethyl acetate – 40% methanol. After the plate has been developed, it was exposed to NH_3 gas followed by brief heating at 100^{o}C before being visualized under ultraviolet light at 366 nm.

Data analysis

The standard curve was created using Microsoft Excel. Data were analyzed with SPSS v.19 statistical software. The analysis included normality test with Shapiro-Wilk test, followed by bivariate analysis (Mann-Whitney or independent t-test) and multivariate (ANOVA) followed by Tamhane's T2 *post hoc* test. Values were significant if $p \leq 0.05$.

RESULTS

NOR Test

NOR test is a rapid behavioral assessment for preliminary screening of cognitive function. This assay stems from the instinctive behavior of rats to explore novel objects, without reinforcing stimuli such as hunger or fear. In this study, treatment with 200 and 800 mg/kg of CA extract significantly enhanced preference index of novel object compared to familiar object (Figure 2).

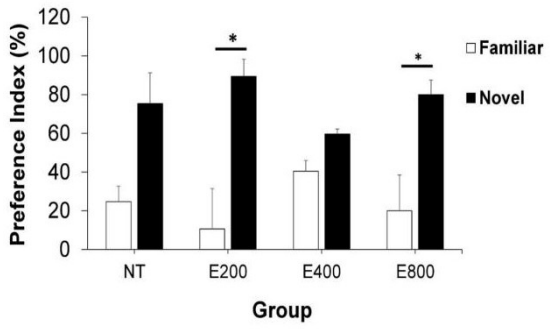

Figure 2. Preference index for familiar and novel object

(NT: no treatment; E200: 200 mg/kg extract; E400: 400 mg/kg extract; E800: 800 mg/kg extract); n = 6 independent experiments. Data are presented as mean ± SEM. *p < 0.05

Acetylcholine content in the brain

Acetylcholine content in the brain was determined by ELISA. The standard curve was created by using 6 concentrations, 6.25 pg/ml, 12.5 pg/ml, 25 pg/ml, 50 pg/ml, 100 pg/ml, and 150 pg/ml. Linear regression was applied, hence the equation y = 0.0024x + 0.0257 with R^2 = 0.9768. Figure 3 shows a significant increase of acetylcholine content in brains of rats treated with 200 mg/kg of CA extract compared to control.

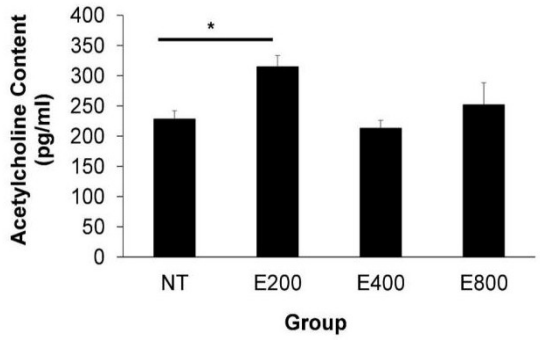

Figure 3. Acetylcholine content in the brain (NT: no treatment; E200: 200 mg/kg extract; E400: 400 mg/kg extract; E800: 800 mg/kg extract); n = 6 independent experiments. Data are presented as mean ± SEM. *p< 0.05

Active compounds in *Centella asiatica* aqueous extract

The aqueous CA extract is approximately 10-15% of dry weight of the herb (Figure 4). Phytochemical tests and thin layer chromatography were performed to detect active compounds contained in the extract. This study confirmed the presence of tannin, phenol, flavonoid and steroid in the extract (Figure 5).

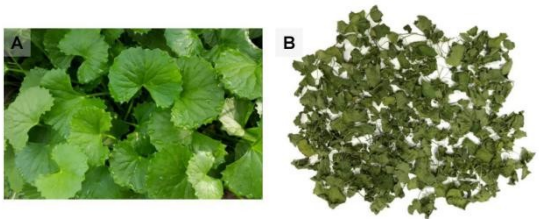

Figure 4. *Centella asiatica*
Fresh (A) and air-dried *Centella asiatica* (B) used in the present study

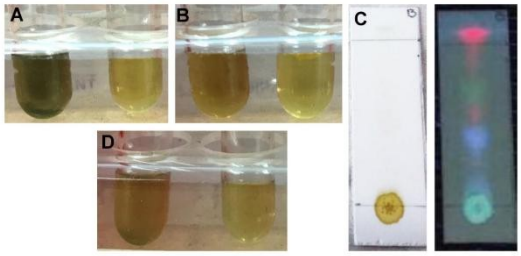

Figure 5. Phytochemical screening of active compounds
Identification of (A) tannin, (B) alkaloid, (C) flavonoid and (D) steroid.

DISCUSSION

To achieve a less-biased evaluation of cognitive performance, this study used the NOR test which does not require external stimuli pressuring the rats to succeed. This is different to the concept of reward and punishment or other external stimuli (e.g. hunger in operant conditioning [18] and T-Maze [19], or fear in elevated plus maze test [20] and passive avoidance test [21]. The test in the present

study also used a relatively short retention interval i.e. 15 minutes, to focus on retention of short term memory. This is certainly different from the Morris water maze test which involves repeated learning, thus allowing stronger memory association as it uses several days of training.[7]

Two identical objects which were introduced in the familiarization phase of the NOR test are termed as 'familiar objects'. Retention time allows rats to establish a recognition memory towards those objects. Therefore, exposure to one familiar object and one novel object during the test phase triggers retrieval of the recognition memory, causing the rats to explore the latter instead.[16,22] Although insignificant, there was a notable increase of preference index for novel objects compared to familiar objects in the non-treated rats. However, this study showed that only those treated with 200 and 800 mg/kg of aqueous CA extract significantly increased the preference index for novel object compared to familiar object. This suggests that this extract specifically enhanced memory retention, similar to that demonstrated by Jared et al.[18]

Previous studies have reported that extracts of CA was effective in improving spatial learning[20,21], and cognitive function in stress-induced.[7,23] These CA extracts contained triterpenoids, such as asiaticoside and madecassoside, which exhibited acetylcholinesterase inhibitory activity in vitro[24] and in vivo[3] and are associated with neuroprotection.[19,25]

However, the present study showed that the aqueous CA extract did not contain triterpenoids, but was positive for tannin, alkaloid, flavonoid, and steroid (Figure 5). The presence of triterpenoids in a CA extract is highly dependent on the location and diverse environmental condition[26–29]. Orhan et al.[28] pointed out that extracts obtained from CA planted in different regions, such as China, Turkey, and India had different concentrations of triterpenoids such as asiaticoside and madecassoside, from most abundant to none. However, they demonstrated that even with little or no triterpenoid content, the extracts obtained from Turkish and India CA showed modest inhibition of butyrylcholinesterase.[28] This selective inhibition is likely to be the cause of increased acetylcholine content in the brain, as both butyryl cholinesterase and acetylcholinesterase hydrolize acetylcholine.[30,31] In fact, previous studies using butyrylcholinesterase inhibitors have demonstrated elevated levels of acetylcholine which was associated with improved cognitive function.[30,31] This possible mechanism in the present study suggests the involvement of compound(s) other than triterpenoids which may contribute to enhanced cognition.

In addition, the presence of flavonoids in the extract of the present study may also contribute to improved cognition. The mechanisms by which flavonoids enhance memory and learning has been well documented, including modulation of synaptic plasticity via PI3-kinase pathways in the hippocampus[32] and an increase of nitric oxide levels which enables vasodilation leading to increased cerebral blood flow.[33,34] Flavonoids have been reported to be responsible for the scavenging activity of aqueous CA extract, which has an IC_{50} of 31.2 µg/ml compared to that of ascorbic acid (2.5 µg/ml).[35] This antioxidant capacity, as well as the modulation of voltage-dependent anion channel (VDAC) in the mitochondria, was cytoprotective for neural cell lines.[36]

CONCLUSION

This study demonstrated that short term memory of healthy cohorts can be enhanced by consuming aqueous CA extract. The recommended dose was 200 mg/kg, as treatment with this particular dose was confirmed with elevated brain acetylcholine content. In absence of triterpenoids, this suggests other indirect mechanisms contributing to this increase, such as modest butyrylcholinesterase activity and the role of flavonoids. The diverse environment where the CA is cultivated is likely to play an important factor in determining the composition of chemical constituents of its extract, prompting necessary qualitative standardization. Further toxicity studies are also required to investigate safety issues in the continuous and intermittent use of this aqueous extract in healthy cohorts.

Acknowledgments

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ACTA BIOCHIMICA INDONESIANA

RESEARCH ARTICLE

THE EFFECT OF INTERMITTENT HYPOBARIC HYPOXIA ON OXIDATIVE STRESS STATUS AND ANTIOXIDANT ENZYMES ACTIVITY IN RAT BRAIN

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ABSTRACT

Background: High altitude can cause hypobaric hypoxia (HH), resulted from the lower barometric pressure and hence partial pressure of oxygen. Hypoxia can lead to a lot of deleterious molecular and cellular changes, such as generation of free radicals or reactive oxygen species (ROS). Increasing of ROS can cause oxidative stress if the antioxidant enzyme does not increase simultaneously. Oxidative damage in brain has toxic effect on cognitive functions.

Objective: In this study, we investigate effect of acute intermittent HH on oxidative stress and antioxidant enzyme activity in rat brain.

Method: Wistar rats divided into 5 groups, consisting control group and four experimental groups which treated to HH. Rats were exposed to simulated HH equivalent to 35.000 feet in hypobaric chamber for 1 minute, repeated once a week.

Results: Level of malondialdehyde and carbonyl in rat brain under acute HH increased at HH exposure (group I) compare to control group. These levels decreased afterward at intermittent HH exposure (group II-IV). Specific activity of superoxide dismutase (SOD) shows increasing level at intermittent HH exposure, especially group IV was increasing of SOD level significantly. The increasing pattern of specific activity of catalase was inversely from SOD pattern, but it still has higher activity in intermittent HH compare to control group.

Conclusion: Brain tissue seems to be able to perform an adequate adaptive response to hypobaric hypoxia after the training, shown by its significantly decreased MDA and carbonyl level and also increased specific activity of SOD and catalase.

Keywords : Intermittent hypobaric hypoxia, Malondialdehyde, Carbonyl, Superoxide dismutase, Catalase

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INTRODUCTION

Hypoxia is a decreased oxygen concentration in tissues and cells, normally caused by reduction in the partial pressure of atmospheric oxygen like in high altitude (hypobaric hypoxia). Due to hypobaric hypoxia, reactive oxygen species (ROS), like superoxide anion (O_2-) and hydrogen peroxide (H_2O_2) are increasingly generated and therefore increased risk of oxidative damage.[1,2] Normal metabolism produced ROS from electron transport chain in mitochondria. Increasing ROS production under hypoxic condition because lack of oxygen can inhibit transfer electron in mitochondria, thereby increasing superoxide radicals at complex III.[3,4]

There is a low oxygen partial pressure (hypobaric) in high altitude, so in this condition also cause lack of oxygen (hypoxia). Air force army and pilots have to routinely undergo training in hypobaric hypoxia conditions. Exposure to hypobaric hypoxia will also cause oxidative stress and affect their health. A study reported that high altitude exposure will increase the reactive oxygen species and decrease the activity of the antioxidant enzyme such as SOD and catalase.[5] Therefore, oxidative stress would be increased because of ROS generation in high altitude.

Oxidative stress is defined as increased free oxygen radicals, ROS and lack of antioxidant substances which are able to neutralize them. ROS generated by a number of processes in vivo are highly reactive and toxic. Some studies reported that ROS exert physiological conditions and play role in signaling pathways regulating cell growth and status redox cell.[6,7] However, when produced in excess, ROS exert unfavorable effects and are able to attack cell macromolecules such as lipid, protein and DNA.[8]

Characteristic of ROS is very reactive to macromolecules in the cells. It can react with lipid and cause lipid peroxidation, results malondialdehyde. ROS also attack protein and DNA resulting carbonyl and 8-OHdG as end product.[8] We can detect these products to evaluate oxidative damage that occurs because of ROS elevation.

Biological systems have accomplished with enzymatic antioxidant as defense mechanisms against the deleterious effects of ROS. Superoxide dismutase (SOD) is the ultimate antioxidant enzymes that protect cell from oxidative damage by ROS. This enzyme converts O_2•- that very reactive to H_2O_2 that less reactive. Further hydrogen peroxide is detoxified become water by catalase enzyme and glutathione peroxidase.[9]

In this study, we want to explore the effect of acute intermittent hypobaric hypoxia exposure on oxidative status in rat brain. This condition is commonly experienced by the air force army through the simulation inside the hypobaric chamber. We want to know whether repeated hypobaric hypoxia exposure will be increasing or decreasing oxidative stress. The parameters of oxidative stress that will be measured are MDA and carbonyl. The antioxidant enzymes that will be measured are specific activity of SOD and catalase.

MATERIAL AND METHODS

Experimental Design

This study was an experimental study carried out at Biochemistry and Molecular Biology Laboratory, Faculty of

Medicine, Universitas Indonesia and Lakespra Saryanto TNI AU. Samples are determined with Federer formula. Twenty five male Wistar rats (6-8 weeks old; body weight 150-250 gram at entry into protocol) were randomly divided into 5 groups (n = 5 per group), consisting control group and four experimental groups. Rats were subjected to acute hypobaric hypoxia by placing them into the hypobaric chamber for one, two, three and four times, respectively. All rats had free access to water and standard rat chow. Water and food consumption was assessed every 2 days. Protocol of hypobaric hypoxia is designed to altitude studies and training especially to train air marshal. Hypobaric chamber is simulated at 35,000 feet for 1 minute to get the effect of acute hypobaric hypoxia. Afterward, the altitude is lowered gradually to 18,000 feet and maintained this level to sacrifice rats and get the brain.

Tissue Preparation

One hundred milligram brain tissues are homogenized in 1 mL PBS. This homogenate is used to measure MDA and carbonyl level. Homogenate for analysis of specific activity of SOD and catalase was added with PMSF as a protease inhibitor.

Measurement of MDA Level

Measurement of MDA level using Wills method. This method is using thiobarbituric acid (TBA) that will react with MDA and form pink color. The homogenate was mixed with trichloroacetic acid (TCA) 20% to precipitate protein. Then centrifuge 6000 rpm for 5 minutes, keep the supernatant. Add thiobarbituric acid 0.67% and incubate at boiling water 95-100ºC. MDA level was appropriate with pink color formed that read by spectrophotometer at 530 nm.

Measurement of Carbonyl Level

Measurement of carbonyl level is using 2,4-dinitrophenylhydrazine (DNPH) that will react with carbonyl substances and form color.[10] The homogenate was added with 10 mM DNPH in 2.5 M HCl and incubated in dark at room temperature. Afterward, add trichloroacetic acid (TCA) 20%, incubate on ice for 5 minutes and centrifuge 10,000 g for 10 minutes, discard supernatant. Add TCA 10%, incubate on ice for 5 minutes and centrifuge 10,000 g for 10 minutes, discard supernatant. Resuspend the pellet by ethanol: ethyl acetate sol (1:1) Add Guanidine HCl and centrifuge 10,000 g for 10 minutes, keep supernatant and read the absorbance at wavelength 360-385 nm. and centrifuge 10,000 g for 10 minutes, discard supernatant.

Measurement of SOD Specific Activity

The specific activity of SOD enzyme was determined using RanSOD® kit (Randox). Superoxide dismutase (SOD) in samples will catalyze the dismutation of the superoxide anion (O_2•) to hydrogen peroxide and oxygen. This method uses xanthine and xanthine oxidase to generate superoxide anion which reacts with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride (I.N.T.) to form a red formazan dye that can be read at 505 nm. The SOD activity is then calculated by the inhibition degree of this reaction because SOD will inhibit superoxide anion generation. Sample was mixed with the reagent and read the absorbance using spectrophotometer at 505 nm after 30 seconds and 3 minutes later. The specific activity is calculated from activity (U/mL) divided by protein concentration (mg/mL).

Measurement of Catalase Specific Activity

Catalase activity is measured by using hydrogen peroxide (H_2O_2) mixed with homogenate.[11] Catalase in sample will convert rapidly hydrogen peroxide into oxygen and water. The decreasing of hydrogen peroxide can be read at 240 nm in 30 minutes (after mixing sample and hydrogen peroxide) and 3 minutes later. The specific activity is calculated from activity (U/mL) divided by protein concentration (mg/mL).

RESULTS

Measurement of MDA level in rat's brain has shown that there was increasing of MDA level at group I (once hypobaric exposure). Meanwhile at group II, III and IV, the level of MDA was decreasing significantly compare to control group ($p<0.001$).

Measurement of carbonyl level has shown that there was increasing significantly carbonyl level at group I compare to control group ($p=0.017$). Afterward, the carbonyl level was decreasing at group II-IV. The decreasing was statistic significantly at group III ($p=0.045$) and IV ($p=0.010$).

Specific activity of SOD in rat's brain under hypobaric hypoxia showed that decreased at group I, but not significant. Later, the activity was increasing group II-IV. However, the significant increasing found at group IV ($p<0.001$).

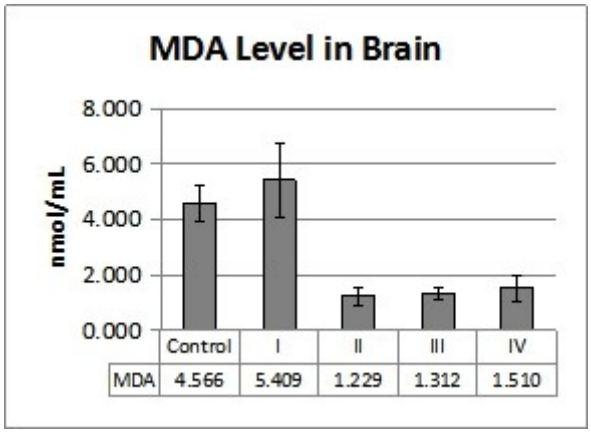

Figure 1. Graphic of MDA level in rat's brain under acute intermittent hypobaric hypoxia

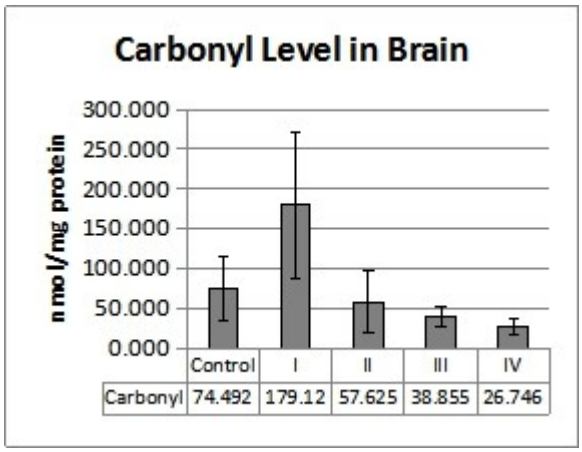

Figure 2. Graphic of carbonyl level in rat's brain under acute intermittent hypobaric hypoxia

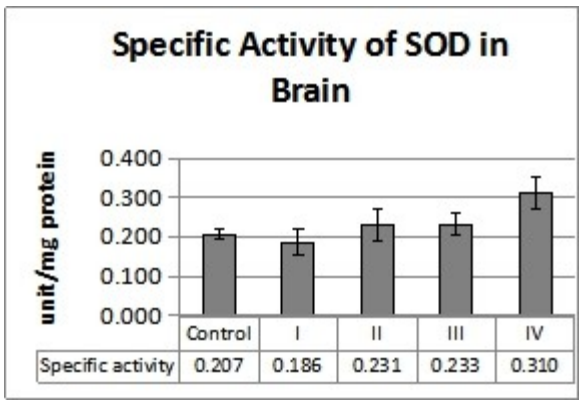

Figure 3. Graphic of specific activity of SOD in rat's brain under acute intermittent hypobaric hypoxia

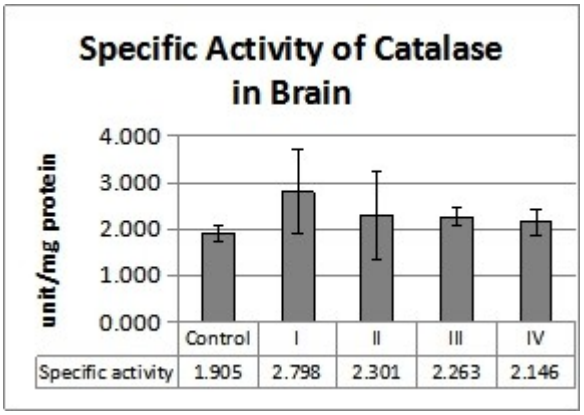

Figure 4. Graphic of specific activity of catalase in rat's brain under acute intermittent hypobaric hypoxia

Results of Specific activity of catalase in rat's brain under hypobaric hypoxia showed inversely with specific activity of SOD. There was increasing of specific activity of catalase at group I, and then the activity was decreased gradually at group II-IV, but the level still higher than control group.

DISCUSSION

Graphics of MDA and carbonyl level have the same pattern. This result showed that at group got first hypobaric exposure lead to increasing MDA and carbonyl level compare to control group. Some previous studies revealed that high altitude exposure will increase oxidative stress conditions, such as lipid peroxidation, DNA damage and nitric oxide production.[12,13]

However, at the group that got intermittent hypobaric exposure, the level of MDA and carbonyl were decreasing. It is because at the group that got repeated hypobaric exposure, occurred adaptive mechanism to hypobaric hypoxia exposure. This can be seen at the parameter of oxidative stress, which decreasing after repeated hypobaric exposure. This result is supported by specific activity of SOD in rat's brain under acute intermittent hypobaric hypoxia. The SOD activity found increasing at group that got intermittent hypobaric exposure. Although there is slightly decreasing SOD activity at group that got the first exposure. The increasing of SOD activity at repeated exposure was meant to combat free radicals (ROS) accumulation because of hypobaric hypoxia exposure.

The specific activity of catalase showed that increasing activity at group that got first exposure. It occurs to against increasing ROS generation at the first hypobaric exposure, before increasing of SOD activity found. Decreasing of catalase activity at group that got repeated exposure is because of adaptive mechanism bearing repetitive hypobaric exposure. The accumulation of ROS under intermittent hypobaric hypoxia was neutralized enough by increasing SOD activity.

Previous study demonstrated that exposure of short-term chronic hypobaric hypoxia will cause the upregulation of transcription factor NRF2 that mediated by oxidative stress. This transcription factor will control the expression of antioxidant enzymes.[14] Another study reported that intermittent hypobaric hypoxia can increase antioxidant capacity in rats brain and protect the neuron cells from oxidative damage.[15]

CONCLUSION

Intermittent hypobaric hypoxia exposure can induce adaptive mechanism against oxidative stress. It is demonstrated by decreasing of MDA and carbonyl level and increasing of specific activity of SOD and catalase in intermittent exposure groups.

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ACTA BIOCHIMICA INDONESIANA

RESEARCH ARTICLE

THE RELATIONSHIP BETWEEN THE UMBILICAL CORD LENGTH AND IT'S DIAMETER WITH THE TOTAL CD34+ AND TOTAL NUCLEATED CELL (TNC) AS A PARAMETER OF CORD BLOOD SELECTION

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ABSTRACT

Background: The stem cell transplantation successful influenced by the quality of the umbilical cord blood which includes the number of CD34 + and Total Nucleated Cells (TNC).

Objectives: The study aims to determine the correlation between umbilical cord length and it's diameter, with the number of CD34 + and TNC cells as indicators of the quality of cord blood storage feasibility. Several other factors as maternal age, gestation period, and infant birth weight also examined.

Methods: Thirty four of umbilical cords from the delivered woman in Dr. Mohammad Hoesin Hospital (RSMH) Palembang were collected from May to June 2018. The length and diameter of the cord were immediately measured after delivery. The evaluated cells were counted with a flow cytometer at Klaster Stem Cell and Tissue Engineering Research Centre (SCTE) IMERI Faculty of Medicine University of Indonesia (FKUI).

Results: Spearman correlation test show that there was no correlation between the length and diameter of the umbilical cord, maternal age, gestational period and infant weight, with the number of CD34 + and TNC cells in the cord ($p > 0.05$). However, the profile analysis indicated that the longer and larger the diameter of the umbilical cord, the higher concentration of the CD34+ and TNC cells.

Conclusion: This study suggested that the younger maternal age, older gestational age, and higher infant birth weight, also normal hemoglobin level, tend to increase the number of CD34+ and TNC cells in the cord blood.

Keywords : CD34+, Total nucleated cells, Umbilical cord blood

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INTRODUCTION

Stem cells are naive cells that can divide and develop into various types of cells in the body and potentially replace various damaged[1] body tissues with two characteristics of self renew and differentiated. There are 3 types of stem cells that can be obtained from the human body: bone marrow, peripheral blood, and cord blood.[2]

Umbilical cord blood (UCB) is the blood that was left over in the placenta shortly after the baby was born and the umbilical cord has been cut. This cord blood is one of the potential sources of hematopoietic stem cells that has a greater number of Progenitor cells compared to the peripheral blood or bone marrow. UCB is also rich in Haematopoietic Stem Cells (HSCs) which will be responsible for the formation of blood and the immune system.[3]

To ensure the quality of cord blood well maintained for a long time, Umbilical Cord Blood Banking (UCBB) was established.[4] Studies indicated that the success of stem cell transplantation is influenced by several parameters such as the number of TNC and CD34+ cells.[5,6] TNC and CD34+ cell numbers reflect the hematopoietic potential of cord blood. Stem Cell transplants success rate are higher when CD34+ cell counts and TNC numbers are high.[7] Accurate CD34+ cell counts are needed to calculate the dosage needed for Stem Cell Transplantation. So, the success of Stem Cell transplantation is very dependent on CD34+ cell count calculations. TNC doses that will provide during transplantation is also a very important factor for the use of hematopoietic stem cells in cord blood transplantation. Higher cost of TNC can increase the success of cord blood transplantation.[8] A number of factors such as the length and diameter of the umbilical cord, maternal age, gestation, hemoglobin of the preterm mother and infant weight at birth may affect the number of TNCs and CD34+ cells.[9]

This study is designed to determine the correlation of umbilical cord length and diameter, and other factors as maternal age, gestational age, pre-maternal hemoglobin and infant birth weight to the number of CD34+ and TNC cells as indicators of the quality of the feasibility of cord blood storage.

MATERIAL AND METHODS

Thirty four of umbilical cords from the delivered woman in Dr. Mohammad Hoesin Hospital (RSMH) Palembang were collected from May to June 2018. Subjects were required to sign an informed consent form before samples were taken. This study was ethically approved by Health Research Review Committee of Mohammad Hoesin Central Hospital and Faculty of Medicine, Sriwijaya University (No.49/kepkrsmhfkunsri/2018)

Cord blood collection and umbilical cord measurement

Hemoglobin levels was measure from venous blood before the subject give birth. Shortly after the baby was born, the umbilical cord that connects the baby and mother is clamped and cut and the umbilical cord that still attached to the mother is disinfected using Povidone Iodine. Next, UCB was collected by obstetricians or midwives that aided the process of delivery within-utero technique. The placenta was removed and the umbilical cord was measured starting from the base of the placenta and then moving towards the cutting site using a gauge. The diameter of the umbilical cord was measured by placing the surface of the

umbilical cord into a caliper and read the diameter on it.

Sample handling

The cord blood that has been taken then immediately sent to the laboratory for sample handling. The sample is centrifuged to obtain a buffy coat and stored in a -80^{0}C freezer until the number of samples is fulfilled.

TNC and CD34+ cells counting

Samples that have been prepared are stained using Trypan Blue and examined under a microscope to see the number of nucleated cells. The potential of existing stem cells then evaluated using the flow cytometry method for TNC and CD34+ cell markers. Each cell will be read and shot by a laser beam one by one through their respective channels. The shot will be read by two detectors to describe the size and content of the cell.

Statistical analysis

The statistical analysis for this study is performed using SPSS 16.0. Since most the continuous data were not normally distributed (based on the Shapiro-Wilk normality test), we used the Spearman correlation analysis test to measure the relationship between the umbilical cord length and diameter with the number TNC and CD34+ cell and the other study variables.

RESULTS

Descriptive statistics were carried out on CD34+ cell counts, TNC, cord length and diameter, maternal age, gestation period, hemoglobin of preterm mothers and infant body weight (Table 1).

Based on the data distribution analysis, there were some outliers in the dataset of each variable on the study as showed by the boxplot diagram below (Figure 1). There is one outlier in the lower side and one outlier in the upper side of the umbilical cord length dataset, 65cm and 28cm, respectively. In the TNC group, there were 2 outliers in the upper side of the dataset group, and one of them with very high TNC count number (3179520 cells/uL) showed by a longer upper whisker on the boxplot. In the CD34+ cell group, there were also 2 outliers on the upper side of the data group, one of them with the CD34+ cell concentration more than 2.5 million cells per uL. The infant birth weight dataset group showed one upper outlier and one lower outlier value, 4100 grams, and 1350 grams, respectively (Figure 1).

The longer and the larger the diameter of the umbilical cord, the higher the concentration of TNC and CD34+ cells, and vice versa. For example, one case with short umbilical cord (28cm) only contained very low number of TNCs and CD34+ cells which were 17760 cells/uL and 100640 cells/uL, respectively. The cases with short umbilical cords may be related to inadequate nutritional intake during pregnancy. A condition where the mother lacks nutritional intake during pregnancy is considered as a factor that plays a role in the process of TNC and CD34+ cell transport from the maternal blood circulation to the umbilical cord which in turn causes low TNC and CD34+ cell concentrations. Further studies might be needed to support this finding.

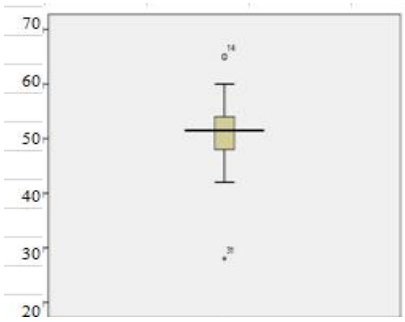
The study showed that there was no correlation between the length and diameter of the umbilical cord, maternal age, gestation period, hemoglobin of preterm mothers and infant birth weight with TNC and CD34+ cells (Table2).

Table 1. Descriptive characteristics of subjects

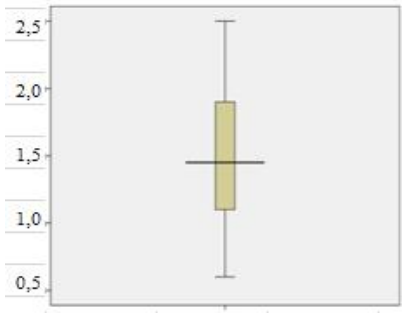
Characteristics	N	Min	Max	Mean value ± SD
Umbilical cord length (cm)	34	28	65	51.4 ± 6.8
Umbilical cord diameter (cm)	34	0.6	2.5	1.4 ± 0.5
Maternal age (years)	34	21	35	28.4 ± 3.6
Gestation period (weeks)	34	29	42	36.7 ± 3.1
HB level (g/dL)	34	8.5	13.2	10.9 ± 1.3
Neonates Birth Weight (gram)	34	1300	4100	2677.5 ± 593.7

Tabel 2. Spearman Correlation Test

Outcome Variables	Predictor Variables	Number of samples (n)	*p value*	Cooefisien Correlation (r)
CD34+	Umbilical cord length	34	0.148	0.253
	Umbilical cord diameter	34	0.543	0.108
	Maternal age	34	0.683	-0.073
	Gestation period	34	0.067	0.318
	Hb level	34	0.685	0.072
	Neonates birth weight	34	0.107	0.282
TNC	Umbilical cord length	34	0.165	0.244
	Umbilical cord diameter	34	0.831	0.038
	Maternal age	34	0.175	-0.238
	Gestation period	34	0.156	0.249
	Hb level	34	0.538	0.109
	Neonates birth weight	34	0.072	0.313



Umbilical Cord Length



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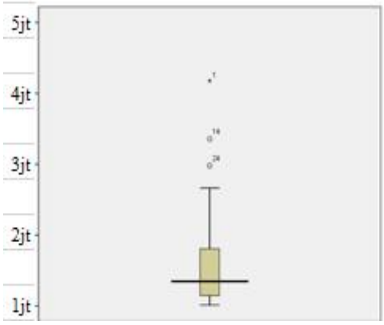
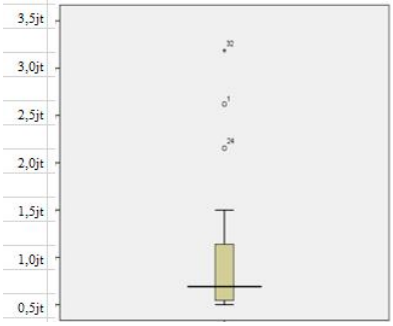


TNC



CD34+ Cell

Figure1. Data distribution of each research variable

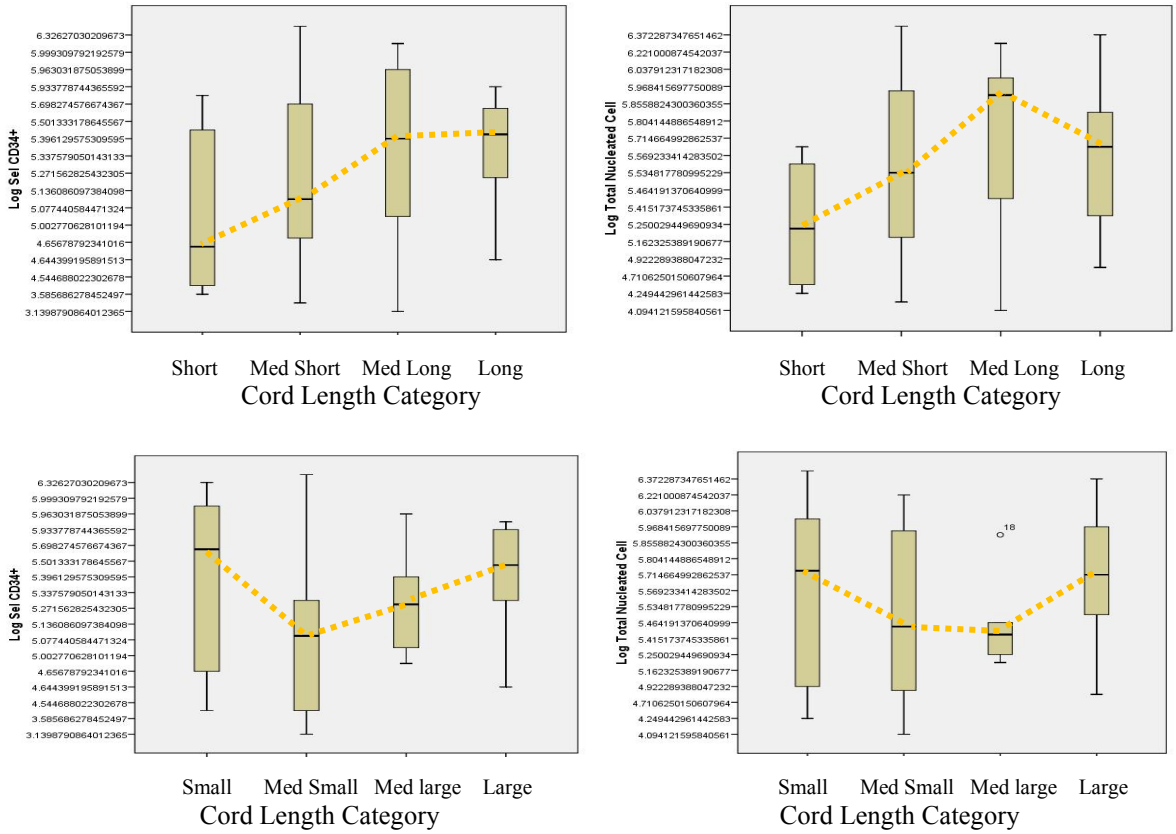


Figure2. CD34+ and TNC cell profiles in each variable

DISCUSSION

The number of TNC and CD34+ cells when collecting cord blood that will be used for storage and transplantation have to be assured exactly because high amounts of TNC and CD34+ cells will provide good transplant results.[8]

The finding of the highest value above was followed by several other supporting data such as 30 weeks gestation, Hemoglobin for preterm mothers 10.3 g/dL, infant weight of only 1600 grams with leukocytes reaching 42.15x10^3/uL, and accompanied by fetal history, premature rupture of membranes. Increasing leukocytes in the mother's blood and the increase of TNC and CD34+ cells in cord blood shows that Stem Cell plays a role in repairing small tissues/organs due to injury or inflammation in the mother's body.[9] In this study, it's look like the UCB with a very high number of CD34+ cells and TNC do not automatically have a higher quality of the stem cell if accompanied by a history of abnormalities in both the mother and fetus. This discovery needs to be further investigated to prove it.

From the boxplot analysis on TNC and CD34+ cell profiles (Figure 2), it shows that the longer the cord, the higher concentration of CD34+ cells and TNC. But when the length of the umbilical cord has exceeded, the TNC concentration will decline. It observed that CD34+ cells and TNC profiles on the umbilical cord diameter are quite fluctuating. In the beginning, CD34+ cell and TNC concentrations were high and then

decrease, but then increases along with the change of cord diameter to be bigger. This result were in line with basic theory about the development of Stem Cells on the umbilical cord. For the maternal age category, TNC and CD34+ cell concentrations will decrease with age, related to the productive age of the reproductive organs and physiological functions. Non-productive age mothers (> 35 years) have a higher risk for pregnancy complications. For the gestational period, both time past (premature) or past time (post term) shows lower CD34+ cell concentration. This is inversely proportional with TNC where TNC concentrations are increases along with mother's pregnancy. This is in line with the research conducted by Nakagawa *et al.* and Wen SH *et al*.[10] In the Hemoglobin category of preterm mothers, TNC and CD34+ cell concentrations seen to be higher as maternal Hemoglobin levels are closer to the normal category. In the infant weight category, the profile of TNC and CD34+ cell concentrations increases along with increasing infant weight at birth, but these will decreases when babies are born in excess weight, which is in line with the research of Mancinelli *et al.* and Phillip J *et al*.[11,12]

There was no correlation between the length of the umbilical cord and the diameter of the umbilical cord to the number of TNC and CD34+ cells, in line with research conducted in Indonesia and Jordania.[13,14] There were no correlation between maternal age with TNC and CD34+ cells. This result was different from the research conducted by Philip J et al,1 related to the difference of samples number. For gestational variables, no correlation was found between gestation and TNC and CD34 cells, in line with research conducted by Urciuoli P *et al*.[15] There was no correlation between the pre-maternal Hemoglobin of the number of TNC and CD34+ cells, different from the study of Djuwantono *et al*,4 related to the difference in isolation techniques from the samples examined. There was no correlation between infant body weight to TNC and CD34+ cell numbers, in line with research by Cannabaro *et al*.[16]

CONCLUSION

In summary, the study found that no correlation appear between the length and diameter of the umbilical cord with the number of TNC and CD34+ cells. However, the concentration of TNC and CD34+ cells is directly proportional to the length and diameter of the umbilical cord, gestational age, hemoglobin levels of the preterm mother, and the infant's weight at birth. Contrast with maternal age, that the older the maternal age, the lower the concentration of TNC and CD34+ cells.

We suggest using the longer and larger UCB for more optimal CD34+ concentration. For more optimal TNC concentration, we recommend using larger diameter of umbilical cord. TNC concentration will decline in excessive cord length, ages and birth weight.

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ACTA BIOCHIMICA INDONESIANA

RESEARCH ARTICLE

HUMAN SERUM FOLATE CAN BE MEASURED USING FOLATE BINDING PROTEIN LINKED TO ENZYME-LABELED PROTEIN LIGAND BINDING ASSAY (ELPLBA) AS WELL AS ELISA

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ABSTRACT

Background: Folate is an important substance used for purine and pyrimidine nucleotide synthesis. One measurement of folate that already establishes is using ELISA (Enzyme-linked immunosorbent assay) method. Folate binding protein is a protein that can bind folate, therefore it considered can be used as a tool that can replace antibody dependent ELISA method.

Objectives: The aim of this research was to create a method for folate measurement in serum called Enzyme-labeled protein ligand binding assay (ELPLBA) by replacing antibody as used in ELISA method with folate binding protein (FBP) that purified from the whey of milk.

Methods: The method is tested using 20 serum samples and compared to ELISA. Folate binding protein was purified from bovine's milk using ammonium sulfate up to 90% saturated, DEAE-cellulose anion exchange chromatography and affinity chromatography. SDS-PAGE and western blot were used to establish the protein band of FBP that has molecular weight of ~25-35 kDa. ELPLBA was arranged with stationary phase using aminohexyl-agarose, and folic acid linked on it using carbodiimide.

Results: The result show there was no significant difference of folate concentration between ELPLBA (14.804 ± 2.795) and ELISA method (13.859 ± 3.638), p = 0.363.

Conclusion: ELPLBA method show similarity for determination of folate in serum which was the same as standard folate measurement (ELISA).

Keywords : Serum folate, Folate binding protein, Enzyme-labeled protein ligand binding assay

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INTRODUCTION

Folate is a vitamin B9 that cannot be synthesized in the body so it is only obtained from supplements, fortified foods, and natural foods.[1] The structure of folate derivatives consists of three parts such as pteridine ring (pterin for folic acid), P-aminobenzoyl, and glutamic acid.[2] The role of folate in the body as a coenzyme is involved in nucleotide synthesis (purine and thymine) and amino acid interconversion, and as an antioxidant for decreasing endothelial damage.[2-4] Folate is required in small amounts (± 400 µg for adulthood), but cannot be replaced by other substances.[5]

Some diseases caused by folate deficiency such as megaloblastic anemia, hyperhomocysteine, and preeclampsia are cured in the presence of a high intake of folate.[3,5,6] Examination of the folate level is necessary to prevent or overcome negative effects resulting from folate deficiency.[7] One of the current methods known to be accurate, sensitive, and relatively fast processing time in folate measurements is enzyme-linked immunosorbent assay (ELISA).[8,9]

Specific antibody requirement for ELISA to detect folate is important as a special protein binding folate, thus its preparation becomes a basic requirement of this method.[2,8,10] Several studies have suggested that the existence of a natural protein which is known as folate binding protein (FBP) can bind to folic acid, which is only obtained from a relatively simple separation method in order to as an alternative to ELISA antibodies.[11-13] Folate binding proteins can be found in fresh bovine's milk with levels of 211 ± 7 nmol / L (n = 10) and molecular weight of 30-35 kDa.[13] The homology percentage of folate binding protein from fresh bovine's milk and folate binding protein from humans is ± 83%.[14]

Based on the potential of folate binding protein from fresh bovine's milk, it can be used to measured folate levels in the human body.[13] Folate binding protein used to measure folate levels in serum in the previous study by Mardiana.[15] However, standard solutions of folic acid are used only in the range of 25-100 ng/mL whereas the normal levels of human serum range from 6 to 20 ng/mL, therefore, Mardiana's technique cannot be used for human folate measurement.[16] Based on the description above, the steps that need to be create to refine the folate measurement technique include: purifying folate binding protein from fresh bovine's milk using ion exchange chromatography and affinity chromatography, validating enzyme labeled protein ligand binding assay (ELPLBA) technique with pure FBP instead of antibody from competitive ELISA as a standard measurement, and then comparing test, to verify between ELPLBA and competitive ELISA technique.

MATERIAL AND METHODS

Before comparison of ELPLBA with ELISA method, the first step was preparation of ELPLBA. It needed to purify first the FBP from whey of bovine milk. The FBP used as capture molecule to bind folate in ELPLBA as same as a specific antibody in ELISA method.

Purification protocol of FBP started by isolation of whey from bovine milk using ammonium sulfate, following by ion exchange chromatography, and affinity chromatography. To ensure the purification result SDS-PAGE and Western-blot were used.

Purified FBP as capture molecule placed in the surface of microplate well. The FBP will bind the serum folate and its competitor avidin-linked folate. The last step was adding the conjugated enzyme biotin peroxidase. The next step was same as in ELISA method measure the absorbance of the product, oxidized chromogen.

Isolation and purification of FBP

Bovine's milk of 2.5L has centrifuged at 2000 rpm then decreasing its pH to form whey by adding $(NH4)_2SO4$. Ammonium sulfate was added to whey up to 90% saturated and then centrifuged 2000 rpm to separate supernatant and precipitate. The precipitate was put inside a cellophane bag for dialysis.[7,11]

Purification of ion exchange chromatography

The DEAE-cellulose phase stationary preparation was prepared according to manufacture instruction (Santa Cruz). The prepared gel was then suspended in 0.02 M phosphate buffer saline pH 7.2 and packed into a chromatographic column with a diameter of 1.5 cm and a length of 15 cm. After the column was ready to use, 1 mL of $(NH4)_2SO4$-free dialysate was added to the column, then eluted with phosphate buffer saline pH 7.2 with concentrations of 5 mM; 20 mM; 40 mM; 60 mM; 80 mM; and 100 mM. Elution fraction each of 2 mL was collected and measured at λ 280 nm. Elution was discontinued and replaced with subsequent phosphate buffer saline concentration when the absorbance was near 0.[7,11,15]

Purification of affinity chromatography

Preparation of stationary phase affinity chromatography using aminohexyl-agarose and folic acid linked using carbodiimide. The yellow gel was decanted and washed with 0.05M NaHCO3. The medium that did not stick to folic acid was blocked by the addition of ethanolamine 1 M pH 7.2 for two hours at room temperature and washed 2-3 times with 0.02 phosphate buffer saline pH 7.2 containing 0.1 M NaCl.[17,18]

DEAE-cellulose ion exchange chromatography fractions pH was changed to be 3 by adding H3PO4 1M solution and followed by acetate buffer pH 3.5 to separate folate from FBP. It was run the following the protocol. The DEAE-cellulose dialysate (second peak) was mixed with folic acid-bound agar resins in a beaker and added with 1 M NaOH solution to adjust its pH around 7.2, stirred for 3 hours at room temperature and replaced into chromatography column with a capacity of 10 mL. After that, the column was eluted with 0.02 M of phosphate buffer saline pH 7.2 and the absorbance of fractions was measured until the fraction that shows absorbance near to 0 at λ 280. Then, column was re-eluted using acetate buffer with pH 3.5 and 0.5 M NaCl. Folate Binding Protein fractions were collected at the second highest peak, and then inserted into a cellophane pouch and immersed in 0.02 M of phosphate buffer saline pH 7.2 in order to neutralize the acidic pH.[7,11,17]

SDS-PAGE and Western blot

The FBP purification was confirmed using SDS-PAGE based on molecular weight and western blot based on antigen-antibody interaction of FBP. [19,20]

Application of FBP in ELPLBA method on folate measurement.

Folate binding protein of 100µL was coated to the microplate and incubated it for 24 hours at 4°C. After incubation and

washing step, 150 µL of 1% BSA was added into each well (blocking), then incubated 2 hours at room temperature. Furthermore, 50 µL of serum was incorporated into each well followed by adding 50 µL of 1 µg/L folic acid-avidin. Subsequently, the microplate was incubated for 1 hour at room temperature and 37°C (for optimization temperature). Next, conjugated enzyme biotin peroxidase was diluted with PBS pH 6.0 (final concentration of 20 µg/mL of), then plated at approximately 50 µl /well in a microplate. Next, the microplate was incubated for 1 hour at room temperature. [2,15]

After incubation and washing, OPD was added (measured maximum absorbance first) 100 µL/well and incubated for 15 minutes at room temperature in the dark. The last, 100 µL of 2 N Sulphuric acid was added to stop enzymatic reaction and light absorption was read using microplate reader at 490 nm.[15]

Sample preparation

This study was conducted using 20 serum blood of normal patients. All patients (both men and women) were about 24-50 years old. The blood obtained from the patient was then collected in an SST tube and incubated for 20-30 minutes to allow clotting process, after that the serum was separated from blood by centrifugation 2000 rpm for 10-15 minutes. Serum was stored in microtube at -20^{o}C. This study was reviewed and ethically approved by Faculty of Medicine Universitas Indonesia Research Committee (No. 790/ UN2.F1/ETIK/2016).

Statistical analysis

Statistical analysis comparative test was performed with independent sample T-test or Mann-Whitney test. The test results in each group will be considered statistically significant at 95% confidence level.[21]

The accuracy test was considered to be accurate with the percentage of recovery was around 80-120%.

$$\%\text{Recovery} = \frac{\text{Measured concentration}_{\text{spiked}} - \text{Measured concentration}_{\text{neat sample}}}{\text{Theoretical concentration}_{\text{spiked}}} \times 100$$

The data's repeatability test was measured from the % CV value which should not exceed 10%, and the regression analysis of the error data was generally measured using independent t-test with 95% confidence level.[22] The linearity of serum sample was considered normal if the percentage of recovery values was still approximately 80%-120%.[23]

RESULTS

The result of FBP isolation from bovine's milk after dialysis that was purified in ion exchange chromatography produced two peaks (figure 1). Then, its purified using affinity chromatography, and show result two peaks. The second peak that eluted with acetate buffer was suspected as FBP (figure 2). The FBP purification result was confirmed using SDS-PAGE and western blot generated 3 bands in the range of 25-35 kDa (figure 3 and 4).

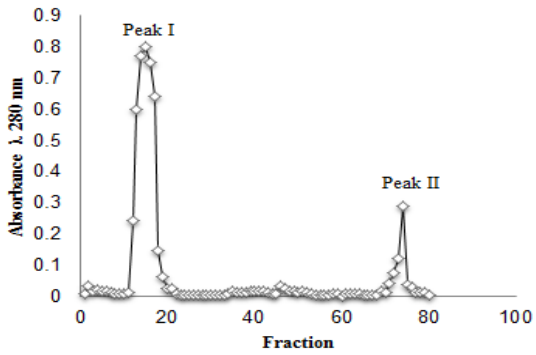

Figure 1. Fractionation with ion exchange chromatography

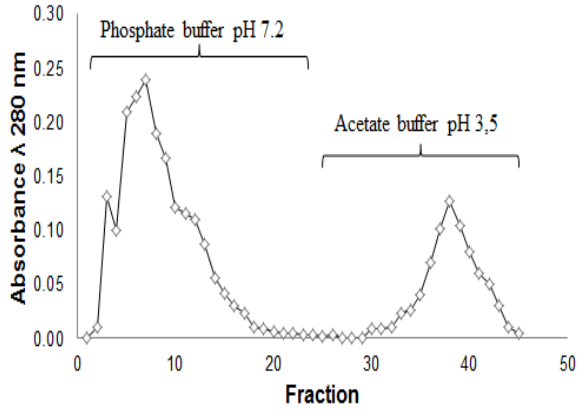

Figure 2. Fractionation with affinity chromatography

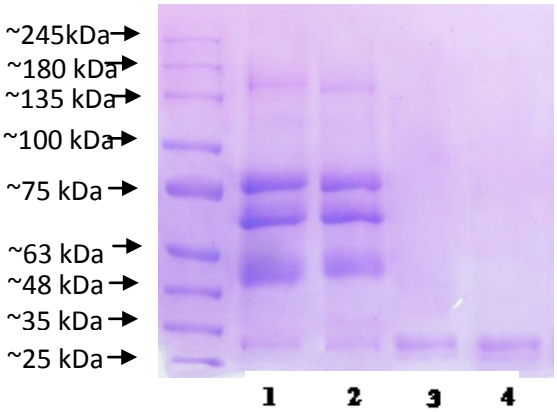

Figure 3. Electrophoresis SDS-PAGE
1: marker, 2,3: dialysate 4,5: second peak eluate

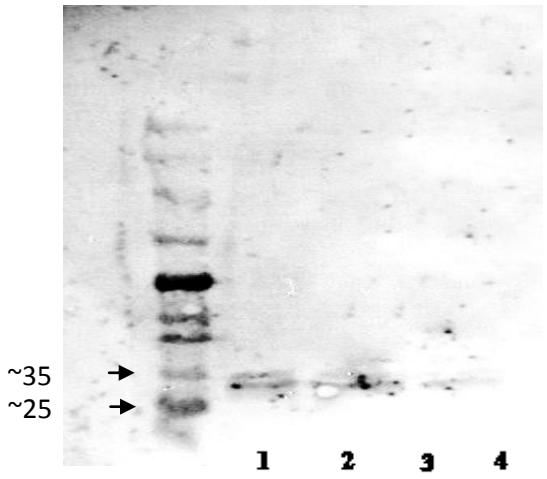

Figure 3. Western blot
1: marker protein, 2,3,4 : second peak of affinity chromatography (FBP) bands.

The temperature optimization techniques of ELPLBA were performed, using purified FBP from the affinity chromatography fraction (data not shown) that also used in competitive ELISA. The optimum temperature that generated based on the highest relative coefficient value was FBP at 25°C with dilution 1/10 that show coefficient of determination (R2) = 0.917.

The repeatability test of ELPLBA on serum folate measurements was performed right after purification in the same day. The result of repeatability analysis (table 1) was 9.8%.

Table 1. Repeatability test ELPLBA

Sample (N=20)	Folate level (ng/mL)		CV (%)	CVstd
	means	SD		
Serum	0,913	0,084	9,8	<10%

The reproducibility test of ELPLBA on folate measurements serum after 5 days was not good, the mean value had significantly different by independent T-test which can be seen in table 2.

Table 2. Reproducibility test ELPLBA

Sampel	N	date	Mean (ng/mL)	SD	p value
Serum	20	5 Mei 2017	14.804	2.79	<0.05
	20	9 Mei 2017	10.307	3.82	

The accuracy and linearity of the ELPLBA on serum folate measurements was performed by the addition of 10 ng/mL of known folic acid concentration (recovery test) can be seen in table 3.

Table 3. The accuracy and linearity of the ELPLBA

Sample	Folate level (ng/mL)	Folate level after spiked	%recovery
Serum a	10.57	19.85a	92.8
1:2		10.67b	107
1:4		5.97 b	120
1:8		2. 98 b	119
Serum b	11.30	19.45a	81.5
1:2		11.61 b	119
1:4		5.534 b	113
1:8		2.4 b	123.58

Comparative test between ELPLBA and ELISA technique showed that both techniques were not significantly different with p=0.363 at 95% confidence level which can be seen in table 4.

Table 4. Comparison test between ELPLBA and ELISA

Sample	N	Technique	mean (ng/mL)	SD	p value
Serum human	20	ELPLBA	14.804	2.795	>0.05
	20	ELISA	13.859	3.637	

DISCUSSION

The isolation of FBP from bovine's milk is aimed to separate the fat and casein through centrifugation and precipitation in order to produce whey. Whey contains many proteins such as immunoglobulin, β-lactoglobulin, α-lactoalbumin, folate binding protein, lactoferrin.[24]

The addition of ammonium sulfate salt to whey is performed in two stages to precipitate the whole casein and whey protein with greater solubility, while the second step to precipitate whey protein with a smaller solubility. The second precipitate resulted in is then suspended and dialyzed using a cellophane pouch with 12 kDa MWCO aiming to restore the protein condition in its initial state.[7,11,25]

The result of dialysis process is further purified by ion exchange chromatography. Folate binding proteins are negatively charged at a neutral pH that can be bound by a positively charged cellulose DEAE. The result of the first peak protein elution is a positive charge protein that does not bind to DEAE cellulose such as lactoferrin, which can come out directly from the column using the phosphate buffer saline eluent pH 7.2. Then, the result of second peak protein elution using phosphate buffer saline pH 7.2 and 0.1 M NaCl was suspected as a negative charge protein containing a folate binding protein. [7,26]

Full purification of folate binding protein from fresh bovine's milk will be done by affinity chromatography based on procedure by Salter et al. The stationary phase used is a crosslinked agarose with folic acid. Crosslinker in the binding of folic acid-agar is carbodiimide.[17,27] The elution result on affinity chromatography shows two peaks, namely the first peak is protein that is not bound by the stationary phase while the second peak is a binding protein with a stationary phase. The second peak is believed to be pure FBP.[7] The result of FBP purification confirmation test using affinity chromatography are similar to Nygren *et al* and Iwai *et al* studies because of glycosylation differences leading to the molecular weight of FBP from ± 25,700 Da to 30,000-35,000 Da.[19,20]

Validity technique used in this research is a precision test (repeatability, reproducibility), accuracy, and linearity. The repeatability test of ELPLBA

technique on serum folate measurements was performed within the same day. Results of repeatability analysis of 9.8%. The % C.V (coefficient of variance) value of repeatability is considered good if the value of coefficient of variance (C.V) is less than 10%.[21]

On the other hand, the reproducibility test of ELPLBA on serum folate measurement obtained using independent T-test indicates poor error in the measurements of serum folate stored within five days. The measurement of serum folate is generally unstable over the long period of time because it is easily degraded to p-aminobenzoylglutamic acid (PABG) affecting the measurement of the technique itself. Eugène H.J.M. Jansen *et. al*. explained that folate storage for 4 days, showing a decrease of stability up to 60% and 83% in the next day.[28]

The accuracy test is measured from the recovery value in the presence of the addition of a certain concentration of folic acid to the sample. The recovery test by addition of folic acid (spiked) for the serum a shows a recovery percentage of 92.9% and the second serum reaches recovery percentage of 81.7%. Based on the recovery test indicates that both serum can be accepted based on acceptable recovery limit (80%-120%).[23]

The linearity of the serum folate sample was performed by the addition of 10 ng/mL of the folate standard (spike) then diluted with a ratio of 1: 2, 1: 4, and 1: 8. On the basis of linearity, the serum response after adding standard folate (10 ng/mL) and diluting on serum (a) still produces a proportional response in the range or the range of the standard curve with a recovery rate range is about 80%-120%. However, the recovery value of serum (b) at 1: 8 dilution does not include the limit of the recovery range leading not to produce an appropriate dilution response in the standard curve range. This may be due to a matrix component (serum protein) interfering serum folate detection at 1: 8 dilution.[23]

The comparative test results between the two techniques showed no significant differences. This indicates that the results obtained from ELPLBA do not conflict with the results obtained by competitive ELISA technique in the measurement of serum folate at a 95% confidence level.[8,21]

CONCLUSION

The method ELPLBA is eligible enough and relatively simple for determination of folate in serum which was the same as standard folate measurement such as ELISA. FBP from bovine's milk can serve as candidate alternative to antibody in ELISA. However, we consider this study only used ELISA method as standard measurement folate and only applied for small amount subject studies so that can't be used as a base for standardization efforts yet and needed next research plan with modifying the sample preparation.

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ACTA BIOCHIMICA INDONESIANA

RESEARCH ARTICLE

EXPRESSION OF APELIN IS RELATED TO OXIDATIVE DAMAGE IN HEART TISSUE OF RATS DURING CHRONIC SYSTEMIC HYPOXIA

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ABSTRACT

Background: Chronic systemic hypoxia is severe environmental stress for the heart and might lead to the development of heart failure. Apelin is an endogenous peptide that has been shown to have various beneficial effects on cardiac function. Apelin appears to have a role to play in the ventricular dysfunction and maintaining the performance of the heart.

Objectives: In the present study we want to investigate the adaptive response of heart tissue to chronic systemic hypoxia and the correlation with apelin expression and oxidative stress in rat.

Methods: An experimental study was performed using 28 Sprague-Dawley male rats, 8 weeks of age. Rats were divided into 7 groups 4 each, namely control group; normoxia (O2 atmosphere) and the treatment group of hypoxia (8% O2) for 6 hours; 1;3;5;7 and 14 days respectively. Body weight and heart weight were measured at each treatment. Ventricular thickness was measured by caliper, Apelin mRNA was measured using real-time qRT-PCR with Livak formula and malondialdehyde (MDA) level was used to assess oxidative stress due to cardiac tissue hypoxia.

Results: Macroscopic exams showed hypertrophy at day 7th. The relative expression of Apelin mRNA in hypoxic heart is decreased at the beginning and then increased, starting from day-7 to day-14. The MDA levels were significantly increased from day-7 and were strongly correlated with relative expression Apelin.

Conclusion: It is concluded that the increase of Apelin expression is related to oxidative stress in heart tissue of rats during chronic systemic hypoxia.

Keywords : Apelin, Malondialdehyde, Chronic systemic hypoxia, Cardiac hypertrophy

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INTRODUCTION

Hypoxia is a pathological condition that characterized by an insufficiency of oxygen supply to fulfill cellular demand. The level of hypoxic injury in the cardiovascular system depends on the hypoxic intensity, the hypoxic stimuli duration and the cardiac tolerance to oxygen shortage.[1] The cardiac important responses to oxidative stress associated with hypoxia include pathological hypertrophy and ventricular remodeling. Cardiac hypertrophy is a maladaptive precursor to heart failure (HF).[2] Cardiomyocyte hypertrophy is induced by a variety of factors such as mechanical load,[3] neurohumoral factors,[4] and pathological mechanisms. Cardiac hypertrophy represents a typical feature of various cardiomyopathies including ischemic heart disease and arterial hypertension.[5] The hypertrophic responses include changes in the quality and quantity of various factors, such as cell size, gene expression, protein synthesis, and sarcomere organization.[6]

Heart failure is a global problem with an estimated prevalence of 38 million people worldwide, and a number that increases with aging population. Heart failure is a deadly and costly disease with a progressive syndrome, characterized by high mortality, frequent hospitalization, and a decrease in quality of life resulting from the inability of the heart to maintain sufficient cardiac output to meet tissue needs.[7] Current treatments mainly slow the development of this syndrome, and there is a need to develop new preventive and reparative therapies. For this case, we feel the need to search for new biomarkers to complement existing biomarkers using animal models. The study of HF requiring animal models that can live where chronic changes in the structure and function of myocardial can evolve and progression of HF and left ventricular dysfunction (LV) can be quantified.

In this study, the conditions of oxidative stress in rats were carried out by normobaric chronic systemic hypoxia, with the aim of developing an experimental model of HF.[8] One of the responses to cardiac stress is an increase in the expression of various peptides that help the heart compensate for stress. Adipose tissue can regulate cardiomyocyte hypertrophy by issuing various bioactive factors, through reactive oxygen species (ROS). The excessive ROS generation in cardiomyocytes has been shown to mediate the hypertrophic response to stretch or other hypertrophic stimuli, such as angiotensin II, endothelin-1, tumor necrosis factor-α (TNF-α).[9,10]

During hypoxia, ROS formation increases and causes oxidative stress conditions that play an important role in the development of left ventricular hypertrophy and HF.[9-11] If ROS formation exceeds antioxidant capacity, ROS produced in ischemic myocardium can directly cause cell death by damaging cell membrane lipids, protein, carbohydrates, and DNA, which leads to qualitative and quantitative changes in the myocardium that causes HF.[12,13] Reaction of ROS with lipid membrane, rich in polyunsaturated fatty acids will cause lipid peroxidation. Lipid peroxidation, which initiated with the presence of hydroxy radicals which results in the production of malondialdehyde (MDA), directly produces oxidative stress. Thus MDA can be measured by thiobarbituric acid test (TBARS test).[14]

Apelin is a new adipocytokine, initially isolated from bovine stomach tissue extracts by Tatemoto in 1998.[15] The apelin precursor is translated as a

77–amino acid preproapelin and the apelin propeptide has many potential proteolytic cleavage sites forming apelin-36, apelin-19, apelin-17, apelin-16 apelin-13, apelin-12 and also the pyroglutamate (Pyr1) apelin-13 subtypes.[15-17] Apelin is a newly endogenous peptide that is a ligand for the apelin receptor, which was described as an orphan G-protein-coupled receptor (GPCR, APJ).[15-18] All apelin isoforms may function through the unique APLNR, their tissue specificity, binding affinity to APLNR and efficacy in APLNR recycling may lead to differential functions of isoforms. It has been shown that apelin signaling pathways are widely represented in the cardiovascular system and it is an important regulator of cardiac function.[19] Apelin and its receptor APJ are expressed in several tissues (stomach, heart, lung, skeletal muscle, etc.) and in the brain, including the hypothalamus.[20] The apelin is expressed in the endothelium via paracrine and endocrine signaling to activate APJ on myocardial cells, endothelial cells, and some smooth muscle cells.[16-21] Apelin has a functional role in cardiovascular development and may also participate in cardiovascular pathological processes.[22-24] In preclinical models, apelin causes nitric oxide-dependent vasodilatation and increases cardiac contractility in rats with normal and failing hearts.[15,25] Foldes *et al*. showed that in human HF Apelin expression levels were higher than those of normal tissue, and suggested that apelin might be involved in the pathophysiology of human HF.[26] Acute Apelin infusion increases cardiac output and lowers blood pressure and peripheral vascular resistance in patients with HF.[27] Foussal et al. reported that administration of exogenous apelin can maintain heart function through reduced oxidative stress and increase catalase activity. This suggests that apelin is a powerful regulator of cardiomyocyte antioxidant defense against oxidative stress in hypertrophic myocardial remodeling.[13]

Tatemoto et al. have described a novel adipokine, produced and secreted by human and mouse adipocytes, apelin,15 and reported that reactive oxygen species (ROS) and oxidative stress may increase the apelin levels.[28,29] The aim of this study is to analyze the adaptive response of heart tissue during chronic systemic hypoxia by investigating the relative expression of Apelin gene in rat and its relation with oxidative stress caused by hypoxia.

MATERIAL AND METHODS

Animal

All procedures were approved by the Ethical Research Committee FMUI No 354/UN.2F1/ETIK/2015 This study was an in vivo experimental study, using 28 male healthy Sprague-Dawley rats weighing 200–250 g. Rats were allowed to access food and water ad libitum. The animals were adapted for at least 7 days before experiments. After a period of adaptation, the rats were divided into 7 groups randomly. Group 1 (Normoxia) was control group within atmospheric air. Six other groups (Hypoxia: H1-H6), were the treatment groups, exposed to hypoxia (8% O2, 92% N2) for 6 hours; 1; 3; 5; 7 and 14 days respectively. All groups were sacrificed at respected time by mean of decapitation after ketamine anesthesia. The thoracic cavity was opened and the heart was quickly excised from the aortic root. Heart tissues were weighed and cut transversely in the subangular region, below the atrial-ventricular junction in two parts.[30] The lower part of the heart is measured by the thickness of the ventricular wall in the thickest part using

caliper (precision 0.01 mm) and then heart tissue placed into microtube frozen immediately and stored at -80ºC for analysis of Apelin expressions and MDA content.

Quantitative real-time RT-PCR

Total RNA was isolated from 25 mg of heart tissue using the Total RNA Mini Kit, Tissue (Geneaid) according to the manufacturer's instructions. Tissue samples were homogenized in a microcentrifuge tube using micropestle. After washing with absolute ethanol, the final RNA was elected in 50 µl of RNase-free water. The purity and concentration of RNA were verified by optical density (OD) absorption ratio OD260 nm/OD280 nm between 1.8 and 2.0 using Varioskan (Thermo Fisher).

Table 1. Primer sequences used by real-time quantitative RT-PCR

Gene	Primer sequences
Apelin	Left: 5'-GTGAAGCCCAGAACTTCGAG-3'
	Right: 5'-CAGCGATAACAGGTGCAAGA-3'
18srRNA	Left: 5'-CGCGGTTCTATTTTGTTGGT-3'
	Right:5'-AGTCGGCATCGTTTATGGTC-3'

The homogeneous cDNA was generated by the reverse transcription of RNA samples using the AccuPower® CycleScript RT PreMix (dN12) (Bioneer) Thermocycler. Real-time PCR was performed using a detector (Exicycler) and then subjected to PCR with SYBR Green I dye in real-time monitoring as the detected PCR products. The PCR solution contained specific left primer and right primer (1 µL each), 5 µl cDNA template, and adjust by DEPC-distilled water to a final volume of 20 µL. The PCR primers designed with Primer3 based on GeneBank were depicted in Table 1. The reaction conditions for amplifying DNA were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15s, 61 °C for 30 s, and 72 °C for 30 s. The mRNA expression was normalized to the expression level of 18s rRNA and was calculated using the following equation: Fold change = $2^{-\Delta\Delta Ct}$

Assessment of oxidative stress by TBARS estimation

To assess the oxidative damage, MDA level (a marker of lipid peroxidation) was measured by reaction with thiobarbituric acid (TBA) methods as described Wills. MDA and TBA react to produce a pink pigment with maximum absorption at 532 nm. 400 µL of homogenate was added 200 µL of trichloroacetic acid (TCA) 20%, then centrifuged. The supernatant was added 400 µL of a solution of TBA. Furthermore, it was incubated in a water bath for 10 minutes at a temperature of 80-100ºC, then cooled to room temperature. The absorbance was measured against a reference blank at 532 nm by spectrophotometer Genesys 10 UV scanning thermoscientific. 1,1,3,3-tetramethoxypropane (Sigma Chemicals, USA) were used as a standard. The levels of MDA in heart tissue were expressed as nmol/mL.

Statistical Analysis

All data were presented as means ± SEM (n = 4 per group) and analyzed using GraphPad software (Version 6, for windows). A one-way analysis of variance (ANOVA) was used to detect statistical differences between groups. A post hoc test (LSD) was performed to determine differences between groups. Significant differences were considered at $p < 0.05$.

RESULTS

Table 1 shows the results of blood gas analysis and hematology. The changes in various parameters as induced by hypoxia, which was expressed as mean ± SEM (standard error of the mean). Table 1 shows the pO_2, pCO_2, arterial O_2 saturation, and HCO_3^- decreased gradually in line with the duration of treatment of hypoxia, while blood pH decreased at the end of exposure. On the other hand, hypoxia led to an increase in hemoglobin, hematocrit and red blood cells (RBC). The increase in all three parameters will continue until the end of the treatment and the concentration or amount to be very high.

Figure 1 shows macroscopic features of the normal and chronic systemic hypoxia cross-section of rats ventricular after hypoxia of 6 hours 3, 5,7 and 14 days respectively. The ventricular wall thickness was significantly increased at right ventricular on 7 days hypoxia and left ventricular on 5 days hypoxia, which indicated an occurrence of ventricular hypertrophy induced hypoxia.

Table 2 shows morphometric evaluation of the normal and chronic systemic hypoxia of rats heart ventricular in hypoxia 6 hours, 1 day, 3 days, 5 days, 7 days and 14 days. A significant increase was observed in both the right ventricular after 7-days of hypoxia and left ventricular after 5-days of hypoxia. The ratio of ventricular thickness (hypertrophy index) of the right ventricular wall was greater than the left ventricular, as seen after 5 days of hypoxia.

Table 1. Blood gases and hematology Values

Parameter	Normoxia	Hypoxia					
		6 hours	1 day	3 days	5 days	7 days	14 days
pO_2 (mmHg)	95.9 ± 2.0	85.1 ± 0.9	64.2 ± 3.5	59.0 ± 2.6*	53.7 ± 0.9*	44.4 ± 2.1*	35.1 ± 1.9*
pCO_2 (mmHg)	40.4 ± 1.3	38.3 ± 0.5	36.2 ± 1.3	33.3 ± 1.0*	31.3 ± 0.8*	29.2 ± 1.6*	23.7 ± 1.1*
Ph	7.40 ± 0.01	7.40 ± 0.01	7.39 ± 0.00	7.39 ± 0.0	7.38 ± 0.00	7.38 ± 0.01*	7.38 ± 0.01*
HCO_3^- (mmol/L)	24.3 ± 1.3	23.2 ± 0.7	22.1 ± 1.1	20.4 ± 0.9	19.1 ± 0.2	17.9 ± 0.5*	14.9 ± 0.6*
O_2 Saturation (%)	94.1 ± 1.5	80.8 ± 1.0	67.5 ± 3.1	60.7 ± 2.7*	58.0 ± 0.7*	55.3 ± 2.1*	52.1 ± 4.2*
Hemoglobin (g/L)	12.01 ± 0.06	12.03±0.08	13.1 ± 0.1	15.01± 0.2*	16.09± 0.1*	17.2 ± 0.2*	19.9 ± 0.2*
Hematocrit (%)	45.3 ± 1.2	46.5 ± 0.4	47.7 ± 1.7	52.5 ± 1.8*	55.4 ± 0.4*	58.3 ± 1.1*	63.2 ± 1.2*
RBC (ml/1000)	6.8 ± 0.1	6.9 ± 0.0	7.1 ± 0.1	8.0 ± 0.0*	8.3 ± 0.1*	8.5 ± 0.2*	9.7 ± 0.2*

Mean values ± SE, * (Significant difference versus normoxia $P<0.05$,
pO_2, pCO_2, O_2 saturation and HCO_3^- fall dramatically, this means that the treatment provided is causing systemic hypoxia. In Advance hypoxia created severe metabolic acidosis (decrease in pH and HCO_3^-) with the compensation for severe respiratory alkalosis (pCO_2 decreased).

Table 2. Evaluation of cardiac morphometric

Parameter	Normoxia	Hypoxia					
		6 hours	1 day	3 days	5 days	7 days	14 days
Right ventricular thickness (mm)	1.36±0.02	1.38±0.06	1.4±0.05	1.43±0.03	1.61±0.02	1.74±0.03*	1.9±0.09*
Right ventricular hypertophy (%)		0.92%	2.75%	4.59%	18.35%	27.52%	39.45%
Left ventricular thickness (mm)	2.23±0.03	2.28±0.03	2.30±0.02	2.34±0.02	2.4±0.02*	2.61±0.06*	2.79±0.06*
Left ventricular hypertophy (%)		2.25%	3.37%	5.06%	7.87%	17.42%	25.28%

Mean Value ± SEM, * significant difference versus normoxia ($p < 0.05$)
Index Hypertrophy (%) = ((Ventricular Thickness Hypoxia - Ventricular Thickness normoxia) / Ventricular Thickness normoxia) x 100%

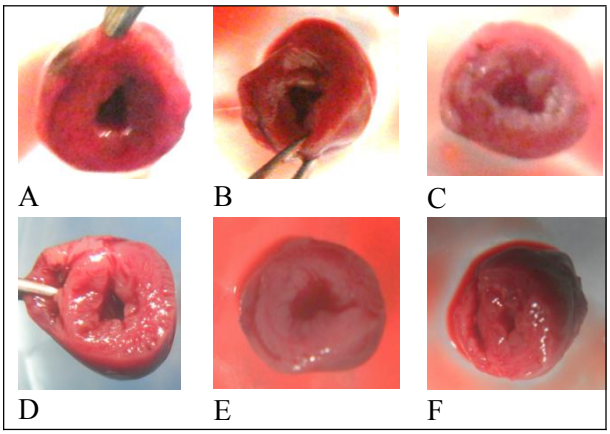

Figure 1. The macroscopic features of the normal and chronic systemic hypoxia cross-section of rats heart ventricular. Transverse sections of the hearts. A. Normoxia, B. 6 hour hypoxia, C. 3 day hypoxia, D. 5 days hypoxia, E. 7 days hypoxia, and F. 14 days hypoxia. The left ventricular and right ventricular wall thickness increases in proportion with the duration of hypoxia treatment.

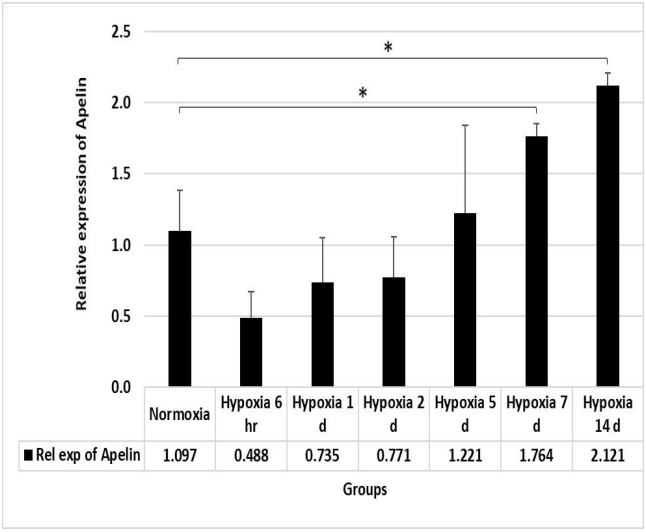

Figure 2. The expression of mRNA Apelin in the heart tissue. There were significant differences between normoxia and the hypoxic groups 7th and 14th days.

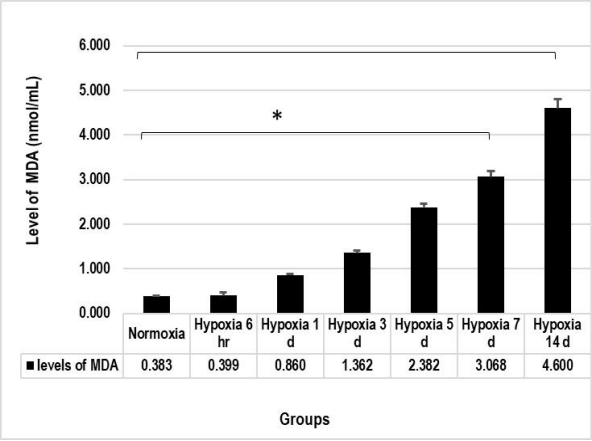

Figure 3. MDA levels in Heart Tissue. There were significant differences between normoxia and the hypoxic groups 7th and 14th days.

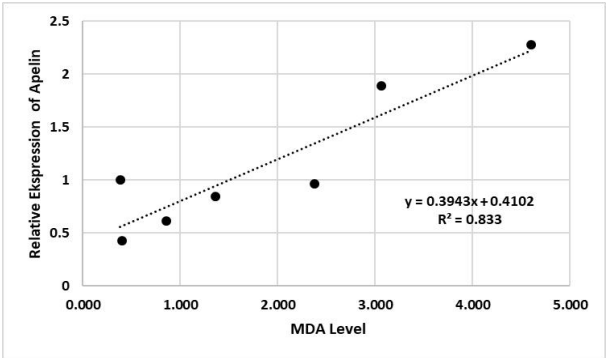

Figure 4. Correlation of MDA levels and Apelin relative expression in heart tissue of rat-induced by systemic hypoxia. The level of MDA and relative expression Apelin is strongly correlated and significant ($r = 0.667$; $p = 0.001$).

Figure 2 shows relative expression of apelin as a result of chronic hypoxia induction, in mean±SE. Product of real-time RT-PCR was detected as fluorescence absorbance of SYBR Green. Threshold of fluorescence curve was set-up to achieve the optimum efficiency of expression. The relative expression of Apelin mRNA in heart hypoxic was decreased at the beginning and dramatically increased with the duration of treatment, starting from 7-days until the end of the treatment period. Total RNA was extracted from the heart, and measured by UV light absorption wavelength of 260 nm.

MDA, a biomarker of cardiac oxidative injury, is a product of lipid peroxidation that can be produced by a variety of oxidative damages. As shown in Figure 3, the hypoxia significantly increased MDA levels at days 7 and 14 ($P < 0.001$) in comparison with normoxia group. The increase of MDA occurred from the beginning (h 6 hours) until the end of the treatment. Figure 4 showed that the increase of Apelin expression is related to oxidative damage in heart tissue of rats during chronic systemic hypoxia.

DISCUSSION

The experimental study conducted in rats which were conditioned to hypoxia for 14 days using 8% O_2 and 92% N_2. All parameters such as blood gases pO_2, pCO_2, O_2 saturation, and HCO_3^- fall dramatically, this means that the treatment provided is causing systemic hypoxia. In Advance hypoxia created severe metabolic acidosis (decrease in pH and HCO_3^-) as the compensation for the shortness of breath due to lack of O_2 breathing accelerated and pCO_2 decreased causing respiratory alkalosis. Witt *et al*.[31] reported that the striking decline from the same parameters has occurred since the first hours of exposure to hypoxia. Comparable results were also reported by other researchers.[1,32] The increased in hemoglobin, hematocrit and RBC occurred as compensation for the decrease of pO_2 level in the network, so that through the increase in all parameters, improved O_2 transport. However, this causes an increase in blood viscosity, making the heart work harder.

Systemic chronic hypoxia causes an increase of ventricular wall thickness. The left and right ventricular wall thickness were increased in proportion with the duration of treatment. Similar changes in cardiac morphometry were also found by Ferdinal *et al*.[32] In physiologic levels, ROS act as signaling molecules in several cellular functions; on the other hand, in pathologic conditions overproduction of ROS have deleterious effects by damages to the several cellular components.[33] An important mechanism that can explain the occurrence of ventricular hypertrophy as a result of hypoxia is oxidative stress as a result of increased ROS production that leads to cell death, either through the mechanism of apoptosis or by necrosis or autophagy. Furthermore, hypoxia causes fibrosis of the vascular endothelium, either as a direct effect or by stimulating the secretion of Angiotensin II are derived from the sympathetic nervous system and resulting in heart.[8]

Apelin is a peptide ligand of the APJ receptor implicated in cardiovascular diseases. Apelin has many functions such as a positive inotropic, vasodilator and diuretic. The apelin and its receptor can be found in the adipose tissue, with higher concentrations in the lung and cardiovascular system.[34] This study reports that apelin myocardial expression

and secretion are activated by hypoxia. We observe an early upregulation of the rat apelin gene expression under hypoxia (8% O_2) in the heart cells. Rokainen *et al.*[28] said the hypoxia increases the apelin expression in cultured cardiomyocytes.

To determine hypoxia-induced regulation of apelin, we measured mRNA expression after treatment of the rats with hypoxia (8% O_2, 92% N_2).for different time periods. Total RNA was isolated from the heart of a rat exposed to normoxic and hypoxic conditions (8% O_2). After 7 days, the apelin mRNA was significantly increased in the heart of the rat exposed to hypoxia. The expression of Apelin mRNA in heart tissue is shown in Figure 2. Hypoxia and various other stimuli induce HIF-1α signaling cascade, and then transcriptionally activate multiple genes.[35]

It has been reported that apelin is present in human plasma and the myocardium.[16,28] In chronic HF due to ischemic heart disease or dilated cardiomyopathy there is an increase the apelin mRNA levels and apelin plasma levels are reported to increase in the early stage of left ventricular dysfunction.[25,36] Ronkainen et al. said the most presumable candidate for the regulation of apelin gene expression would be hypoxia-inducible factor-1 (HIF-1).[28] HIF-1 is activated in hypoxia due to the prevention of oxygen-dependent HIF prolyl-4-hydroxylase (HIF-P4H)-mediated proline hydroxylation, which in normoxia targets the HIF-1 subunit for ubiquitination and proteasomal degradation.

Hypoxia leads to increased formation of free radicals. MDA is produced in the process of lipid peroxidation by ROS, thus that the measurement of concentration, is used as a biochemical marker of oxidative stress. In hypoxia, ROS production in the respiratory chain increased particularly superoxide anion, as a result of leakage of electrons, resulting in a partial reduction of oxygen. In this study, the treatment of hypoxia comparable to the increased concentration of MDA. The high concentration of unsaturated fatty acids in cell membranes makes cell membranes the main target of free radicals that are formed lipid peroxide with solving the main results in the form of MDA. We demonstrated that the level of MDA in heart tissue had increased since day-1 of hypoxia. The MDA levels increased with the length of exposure to hypoxia and reach the maximum level at the day-14 with a significant increase on day-7 and day-14 of hypoxia The increased level of MDA in heart tissue also proved by Folden *et al*.[37] which found that oxidative stress caused to an increase in MDA level. Increased ROS formation from mitochondria will trigger the redox signaling cascade. ROS will activate signaling pathways and in turn activate transcription factor and expression of target gene.[2,38,39]

Correlation between MDA of rat heart with relative expression of mRNA Apelin of heart tissue got result of correlation coefficient equal to 0,667 which showed a strong positive correlation. This shows that Apelin is expressed under hypoxia/oxidative stress. This is consistent with the increase in MDA concentrations were positively correlated strongly to increase the Apelin relative expression.

CONCLUSION

We created an experimental rat heart damage model with chronic systemic hypoxia. This damage is supported by a significant increase in the relative

expression of Apelin mRNA in chronic systemic hypoxia starting from the 7th day. This shows structural damage due to pressure overload in the ventricular cavity and the ventricular wall stretching resulting in increased ventricular wall thickness. Induction of chronic systemic hypoxia causes an increase in formation of MDA in rat heart tissue because the formation of ROS increases during hypoxia. This study shows that Apelin mRNA levels can be candidates as biochemical markers of heart damage.

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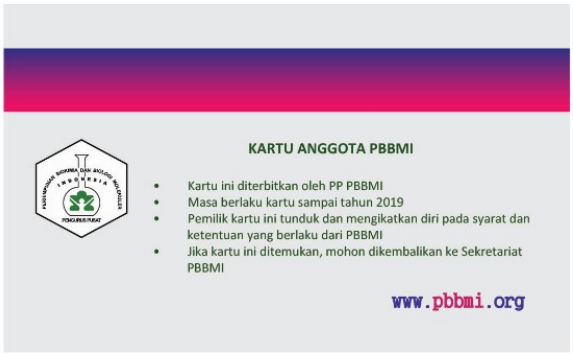

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