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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.

1. Format for publications

Authors should mention the type of the submitted article, Research Article, Short Communication or Review Article.

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.

Editor makes decision about accepted papers as soon as possible. All manuscript are handled electronically troughout the consideration process. Authors will be informed within a week if the paper is not being considered.

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All manuscript should by typed in 1,5 line spacing and 12 point of Times New Roman font in A4 paper with margins 2,5 cm on all four sides. Use line numbers for all manuscript. Page numbers should be located at the right bottom. Tables and figures supporting the reports must be grouped and placed at the end of the manuscript (after the references).

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:

- Title page
- Author's information
- Abstract
- Keywords
- Introduction
- 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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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.

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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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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

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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
Generally, units must be abbreviated according to the International System of Units (SI units: http://www.bipm.org/en/home/). It

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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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.

10. Example

Figure template using scale bar

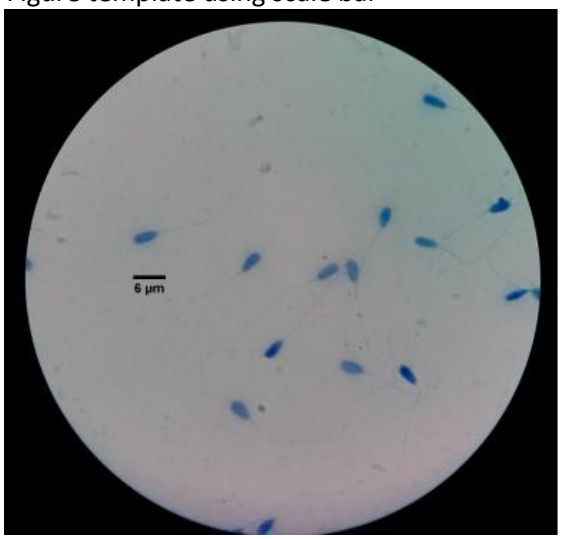

Figure 1. Sperm assay using *Halomax* kit to detect DNA fragmentation

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

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RESEARCH ARTICLE

STUDIES ON BIOSURFACTANT PRODUCED USING *Exiguobacterium profundum*

NA Setiani[1*], W Octaviyani[1], S Hamdani[1], I Mardiah[1]

[1]*Department Biotechnology, Pharmacy, Sekolah Tinggi Farmasi Indonesia, Bandung, Indonesia*

***Corresponding author : nur.asni@stfi.ac.id**

ABSTRACT

Background: The manufacture of pharmaceutical preparations generally adds surfactants. Microbial biosurfactants can be an alternative because biodegradable and have antibacterial properties.

Objective: This study aimed to examine the biosurfactant activity of *Exiguobacterium profundum*.

Methods: Hemolysis and spreading oil tests were performed as an initial screening. Biosurfactant production was carried out by growing bacteria on oil-enriched media with shaker system for 7 days. Biosurfactant activity can be seen from the emulsification index, while the characterization of biosurfactant were used thin layer chromatography and antibacterial qualitative testing.

Results: *Exiguobacterium profundum* could spread the oil layer and form micelles. The emulsification index on days 0, 1, 3, 5, and 7 showed percentage in sequence 44.83%, 48.28%, 48.28%, 40%, and 43.75%. The result of TLC showed lipopeptide group which is marked with red stain with ninhydrin appearance. Antibacterial testing using *Escherichia coli* showed the formation of clear zones around the disk paper.

Conclusion: The biosurfactant produced by *Exigoubacterium profundum* can be classified into lipopeptide group which has antibacterial activity against gram-negative.

Keywords : Antibacterial, Biosurfactant, Emulsification, *Exiguobacterium profundum*, Lipopeptide

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INTRODUCTION

Surfactant is an amphiphilic chemical compound in which hydrophilic and hydrophobic properties exist in one molecule so that it has the ability to reduce the surface tension of a fluid.[1] Surfactants are widely used in industry as an emulsifier, corrosion inhibition, foaming, detergent, dan hair conditioning. The use of synthetic surfactants is considered less profitable because it cannot be degraded naturally (nonbiodegradable) and are of high toxicity and inhibit the degradation process by microorganisms.[2] To reduce these effects of surfactants need to be developed the alternate compound that can be degraded naturally (biodegradable), one of them by using biosurfactants.

Biosurfactants are amphiphilic compounds produced by microbes on cell surface and synthesized extracellular, which can reduce the surface tension between two phases.[3] Biosurfactants from microorganisms have stable chemical properties, renewable, environment-friendly, and low toxicity so that it can be applied in the pharmaceutical industry.[4] In the pharmaceutical field, biosurfactants are used as emulsifiers, foaming agents, wetting agents, and moisturizers.[3] Because of many benefits of biosurfactants, it needs to explore the potency of biosurfactant-producing microorganisms. Preliminary tests and biosurfactant activity tests were carried out on the bacterium *Exiguobacterium profundum*, marine bacterium that has a wide temperature and pH range so that it can adapt to extreme environments. *Exiguobacterium* is a potential genus that is widely used in industry and agriculture. Several studies have shown that the bacteria can be applied in bioremediation and toxic degradation.[5] However, there is no exploration related to its use as a biosurfactant.

MATERIAL AND METHODS

Rejuvenation of *Exiguobacterium profundum*

Exiguobacterium profundum is a bacterial isolate collection in the Microbiology Laboratory Sekolah Tinggi Farmasi Indonesia. Working culture was made by rejuvenating the bacteria into some Nutrient Agar slant by streak method. Then, inoculums were incubated at 37°C for 24 hours.

Screening of biosurfactant-producing bacterial isolate

Biosurfactant screening of *Exiguobacterium profundum* was carried out through hemolysis tests on blood agar and oil spreading techniques. Sterile fresh horse blood (1.5 ml) was added to the Erlenmeyer flask containing 100 ml of sterile Blood Base media. After that, the medium was poured into a sterile petri dish until it solidified. Bacterial isolate was inoculated by streak method on blood agar and incubated at 37°C for 48-72 hours.

The oil spreading technique was conducted by pouring 30 mL of distilled water into a petri dish, coconut oil (1 mL) was pipetted and dropped in the middle. Next step, 20µL of bacterial culture was added in the middle of the oil layer. Emulsion and clear zone formation were observed.

Biosurfactant production

Exigoubacterium profundum bacterial suspension (1 mL) was inoculated into 100 mL of Nutrient Broth media containing 3.3 mL of coconut oil. Next, it was incubated at 25^{o}C for 7 days with a shaker system. Observations were

made at T0, T1, T3, T5, and T7.[6] Separation of biosurfactants and bacterial cells were carried out by centrifugation at 3600 rpm for 20 minutes and the supernatant was taken.

Emulsification test

Exiguobacterium profundum suspension (2 mL) and 2 mL of coconut oil were added into test tube, then vortex for 2 minutes and allowed to stand for 24 hours.[7] Emulsification index was calculated by the equation :

$$\text{Emulsification index (E24)} = \frac{\text{height of emulsified layer}}{\text{height of the liquid column}} \times 100\%$$

Characterization of biosurfactant

The biosurfactant characterization was done through thin layer chromatography and antibacterial activity. Cell-free supernatant was spotted on TLC and eluted using chloroform, methanol, and water in a ratio (65: 25: 4 of volume). The results formed were visualized with UV 254nm, 366nm, and sprayed with ninhydrin.

Antibacterial activity was carried out using the paper disc method. Suspension of Staphylococcus aureus and Escherichia coli were inoculated using the pour method on MHA (Mueller Hinton Agar) media, homogenized, and allowed to solidify. Paper disc containing 20 µl biosurfactant were placed on top of the media layer, incubated at 37º C for 24 hours, and the formation of inhibition zones around the disc paper was observed. The positive control used chloramphenicol 250 µg/50µL[8], while the negative control used sterile distilled water.

RESULTS

Screening of biosurfactant-producing bacterial isolate

Screening of biosurfactant-producing through hemolysis and oil spreading test with the results in Figure 1 was done. The results of hemolysis tests on blood agar were negative because there were no clear zones arranged based on inoculum streak, while the oil spreading technique test showed positive results with the formation of emulsions and clear zones from oil drops of bacterial culture.

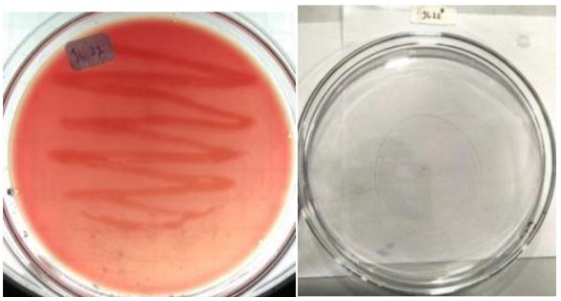

Figure 1. Hemolysis and oil spreading test of *Exiguobacterium profundum*

Emulsification test

There were five observation points for measuring the emulsification index with the biggest results on Day 1 and Day 3 of 48.28% according to Figure 2. The emulsification index decreased on day 5 and increased again on day 7.

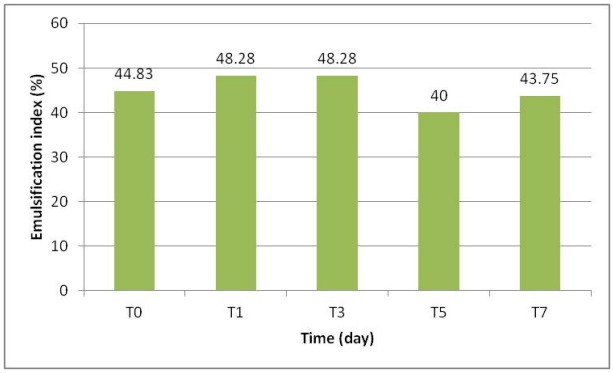

Figure 2. Emulsification test results

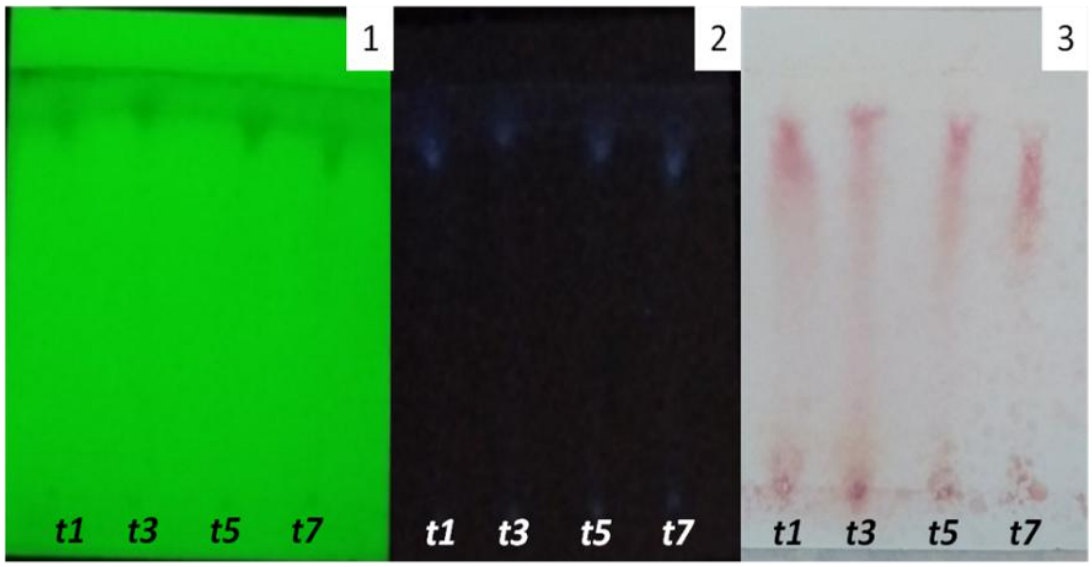

Figure 3. TLC results of the *Exiguobacterium profundum* supernatant using the mobile phase of chloroform: methanol: water (65: 25: 4 of volume) under UV 254nm (1) 366 nm (2) and the appearance of Ninhydrin spots (3).

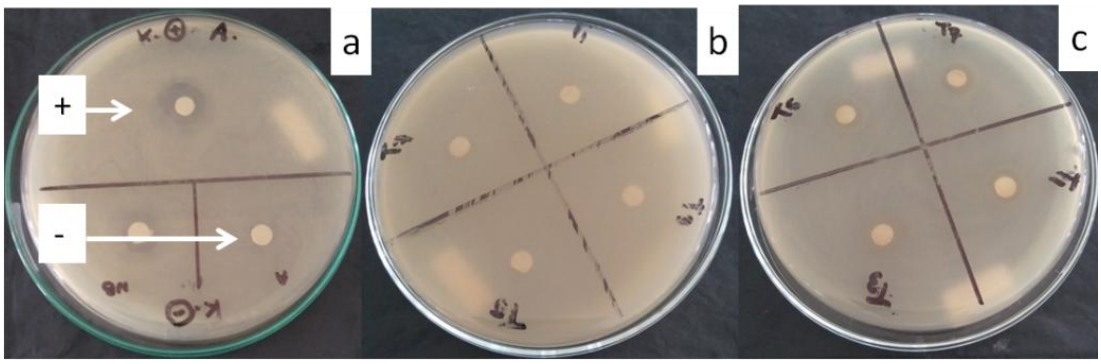

Figure 4. Antibacterial activity test of crude surfactant from *Exiguobacterium profundum* on four observation points (Day 1, Day 3, Day 5, and Day7) a) positive and negative control (b) *Staphylococcus aureus* c) *Escherichia coli*

Characterization of biosurfactant

The results of thin layer chromatography in Figure 3 showed the formation of red spot after being sprayed with the ninhydrin reagent. The antibacterial test in Figure 4 showed that biosurfactant could not inhibit the growth of *Staphylococcus aureus* (Gram-positive) because there was no clear zone around the disc paper, but it could inhibit the growth of *Escherichia coli* (Gram-negative) as indicated by the formation of a clear zone around the disc paper.

DISCUSSION

The manufacture of pharmaceutical preparations generally adds surfactants. Microbial biosurfactants can be an alternative because biodegradable, non-toxic and have antibacterial properties. Based on result, *Exiguobacterium profundum* has the potential to be a source

of biosurfactants. This was proven by oil spreading technique test and emulsification index. Oil spreading technique as initial screening tests showed the oil layer was emulsified and forms micelles because the hydrophobic and hydrophilic parts of the supernatant coalesce thereby reducing surface tension.[9]

Emulsification index test is one method to determine the character of biosurfactant production. *Exiguobacterium profundum* produced an emulsification index of 48.28% which was classified as a good biosurfactant. The higher emulsification index value has better surfactant properties.[10] The ability of bacterial emulsification was influenced by the structure and components possessed by biosurfactants.[11] This ability facilitates the uptake of hydrophobic substrates by cells.

Biosurfactants produced by microorganisms categorized into high and low molecular mass. The main classes of biosurfactants are glycolipids, phospholipids, lipopeptides, and polymeric biosurfactants.[12,13] Characterization using thin-layer chromatography showed that *Exiguobacterium profundum* produced lipopeptide biosurfactants. This indicated by the appearance of red spots after being sprayed with the ninhydrin reagent.[14] Lipopeptide is a low molecular mass biosurfactant component that has broad-spectrum antimicrobial activity and is currently applied in industry, cosmetics, and pharmaceutical.[13] It is not only decreases surface tension but has bioactivity as an antibiotic. Biosurfactants of this group can inhibit the attachment of pathogenic microorganisms to the surface at the site of infection and destroy bacterial colonization.[15]

Further testing of antimicrobial activity showed the biosurfactant produced by *Exiguobacterium profundum* only has antibacterial activity against *Escherichia coli*, gram-negative bacteria. The chemical composition and microbial origin affect biosurfactant activity.

CONCLUSION

Based on the results of research that has been done it can be concluded that the *Exiguobacterium profundum* can produce biosurfactants which belong to the lipopeptide group and have antibacterial activity against gram negative bacteria. Further research needs to be done on optimizing the biosurfactant production of *Exiguobacterium profundum* on various factors such as carbon source, pH, temperature, aeration, and inoculum concentration.

Acknowledgment

We thank Yayasan Hazanah for providing research funding support.

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ACTA BIOCHIMICA INDONESIANA

RESEARCH ARTICLE

A SIGN OF ACUTE INFLAMMATION IN TYPE 2 DIABETES MELLITUS PATIENTS IN KOTABARU AND KALIBARU SUBDISTRICTS, BEKASI

R Amelia[1*], N Arshita[1], SN Fajriah[1], CVD Astuti[1], IN Fitri[1]

[1]*Departemen of Medical Laboratory Technology, STIKes Mitra Keluarga, Bekasi, Indonesia*

***Corresponding author :** riacaramel@gmail.com

ABSTRACT

Background: During the development of chronic type 2 diabetes mellitus (T2DM), inflammatory signals are elevated which can cause microvascular damage. C-Reactive Protein (CRP) is one of acute phase proteins stimulated under inflammatory conditions and creatinine is a waste product used to measure the glomerular filtration rate (GFR). Both of these compounds are considered as biomarkers of acute kidney damage among people with T2DM.

Objective: The purpose of this study was to determine relationship between CRP and creatinine levels in T2DM patients.

Methods: We conducted analytic cross-sectional study in Kotabaru and Kalibaru sub-districts, Bekasi, from January until February 2019. Creatinine was measured using the jaffe method and CRP was measured using a latex agglutination technique. The correlation between CRP and creatinine was analyzed with Spearman test.

Results: Spearman correlation test from 55 samples showed a weak positive correlation ($r = 0.289$; $p < 0.05$) between CRP levels and creatinine levels. These results indicate that high CRP levels are directly proportional to creatinine levels in the serum of T2DM patients. Creatinine and CRP levels can be used as clinical parameters as biomarker for acute microvascular damage in nephron cells that can develop into complications due to T2DM.

Conclusion: There was a significant, weak positive correlation between CRP levels and creatinine levels in T2DM patients in Kotabaru and Kalibaru districts, Bekasi

Keywords : C-Reactive Protein, Creatinine, Inflammation, Kidney, Type 2 Diabetes Mellitus

Received Dec 10, 2019 ; Revised Jan 28, 2020 ; Accepted Jan 30, 2020

INTRODUCTION

Globally, the number of people with diabetes mellitus has quadrupled in the past three decades, and diabetes mellitus is the ninth major cause of death. Asia is major area of the rapidly emerging T2DM global epidemic, with China dan India the top two epicetres.[1] Indonesia was ranked as the seventh highest diabetes prevalence, with 10-20 million of total number of adults (20-79 years) were estimated to be living with diabetes mellitus and is predicted to increase to sixth with 16.2 million diabetics in 2040. Type 2 diabetes mellitus is caused by metabolic disorders and insulin resistance.[1,2] Insulin resistance is a condition of decreased responsiveness of cells or tissues to insulin. As a result of decreased cell response to insulin, glucose in the blood can not be taken into cell, causing hyperglycemia. Hyperglycemia causes cells to produce superoxide oxidants (O_2^-) which can cause cell damage. As a sign of inflammation, inflammatory cytokines such as IL-6 will be activated during cell damage.[3]

Cytokines IL-6 is a proinflammatory cytokine produced by liver cells that can stimulate acute phase proteins such as C-Reactive Protein. C-Reactive Proteins (CRP) can bind to damaged cells using phosphatidylcholine binding site and the membrane binding process can initiate the complement immune response and assists the clearance of apoptotic cells.[4] CRP can activate the classical pathway complement system with C1q molecules and alternative pathways with C3b molecules.[5] In addition, CRP can stimulate the production of proinflammatory cytokines such as IL-6, IL-4, TNF-α and IL-17.[6] The presence of proinflammatory cytokines can increase ability of phagocytic cells such as neutrophils, macrophages to clear destroyed cells in inflamed area.[7] Therefore, an increase in CRP levels is used as a sign of inflammation in the body. If this inflammatory state continues in people with type 2 diabetes mellitus, it can develop into organ damage due to the inflammatory response.[8] Changes in extracellular matrix protein synthesis in conjunction with advanced glycation end-products cause oxidative stress that can lead to complications.[9]

The complications of diabetes mellitus have been divided into macrovascular complications, for example cardiovascular disease (CVD), and microvascular complications, for example nephropathy, retinopathy, and neuropathy.[1] Diabetic kidney disease develops in approximately 40% of diabetic patients.[10] One of the earliest organs to be damaged in people with type 2 diabetes mellitus is the kidney.[8] The natural history of diabetic kidney disease includes glomerular hyperfiltration, progressive albuminuria, reduce glomerular filtration rate (GFR), and finally, end-stage renal disease (ESRD). Metabolic changes associated with diabetes induce to glomerular hypertrophy, glomerulosclerosis, tubulointerstitial inflammation and fibrosis.[10]

Kidney is an organ that functions for excretion of metabolic waste in urine while reabsorption of necessary molecules such as glucose. Hyperglycemia increases blood viscosity thus increases kidney burden to filter and excrete metabolic waste from blood. Disruption of kidney function can be identified by creatinine levels. Creatinine is a breakdown product of creatine phosphate used as a parameter of kidney damage.[8] Therapy has been available to reduce the risk of complications in diabetes kidney disease.

Screening of CRP and creatinine levels in diabetic patients who have not been affected by kidney damage can be used as a preventive measure to reduce the risk of diabetic kidney disease. C-Reactive Proteins can indicate inflammation in diabetics in general and creatinine levels can indicate damage to the kidneys, so that both parameters can be used as initial biomarkers to detect complications in diabetics.

MATERIAL AND METHODS

We conducted an analytic cross-sectional study. The sample was selected using a purposive technique. The research was conducted on behalf of Dinas Kesehatan Kota Bekasi with letter number 070/69B/Dinkes.SDK. The study sample was 55 patients with inclusion criteria such as people with type 2 diabetes mellitus, aged > 36 years, and residents of Kotabaru or Kalibaru sub-district, Bekasi. The specimen used was serum.

C-Reactive Protein was measured using latex agglutination method (CRP AIM test kit) with a cut-off value of 0.8 mg/dL. C-Reactive Protein examination was carried out in two steps. Firstly we conducted a qualitative examination. If the qualitative CRP examination showed positive result, analysis then proceeded with a semi-quantitative examination to determine CRP level.

Qualitative CRP analysis was conducted by dropping 50µl of undiluted serum into the circle on the glass slide with positive and negative controls on the other circles. The serum and controls were mixed with one drop of previously homogenized the AIM CRP Latex Test reagent. The mixtures were homogenized using a stirring rod then shaken for 3 minutes. Positive results are indicated by agglutination in the sample. The semi-quantitative CRP examination aims to determine the levels of serum CRP. First, the serum sample was diluted by stratified dilution. Then, 50µl of saline was dropped in circles 1, 2, 3, and 4. Fifty µl of the serum sample was dropped in circle 1. Solution in circle 1 was mixed, and then 50µl of liquid in circle 1 was transferred to circle 2 and so on. The last dilution series liquid was discarded. Air bubbles formation was avoided during the dilution. After sample dilution was ready, 50µl of AIM CRP reagent was mixed into each circle and shaken for 3 minutes. Positive results are indicated by agglutination in the last circle with titer 1 (1: 2), circle 2 (1: 4), circle 3 (1: 8), and circle 4 (1:16). If the final agglutination is shown in circle 3, then the way to determine serum CRP levels is 1: 8 multiplied with a reagent cut-off value of 0.8 mg / dL with a result of 6.4 mg / dL.

Creatinine was evaluated using the Semi-Auto Chemistry Analyzer BA - 88A based on the standards of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). Assessment criteria were based on the Decree of the Minister of Health of the Republic of Indonesia Number 1792/MENKES/SK/XII/2010 using the Jaffe method. Principle of the reaction was creatinine reaction with alkaline picrate solution to form a reddish-orange complex. The intensity of the resulting color is directly proportional to the concentration of creatinine in serum and can be measured photometrically at λ 500-560nm. Two test tubes was prepared for creatinine measurement, labelled as blank and sample tubes. Ninety µl of reagent A was transferred into both tubes. Then, 9µl of aquadest was transferred into the blank tube while the 9µl of serum was transferred into the sample tube. Tubes

were homogenized and incubated for 1 minute at 37ºC. After that, 90µl of reagent B was transferred into both tubes. Tubes was further homogenized and incubated at 37ºC for 30 seconds. Results were read using the semi-auto chemistry analyzer BA - 88A based on kit creatinine Mindray. The normal range of serum creatinine is 0.6-1.2 mg/dL for man and 0.5-1.1 mg/dL for woman.

The data was presented descriptively. To find out the correlation between CRP and creatinine, statistical tests were performed using the Spearman correlation.

RESULTS

Table 1 shows that respondents were predominantly female (78%) with the mean age of 56 ± 7.7 years. The median of C-reactive Protein in type 2 diabetics was <0.8 mg/dL and creatinine level was 0.55 mg/dL. Both of these data were not normally distributed, so we analyzed the correlation between CRP and creatinin levels using Spearman test.

Tabel 1. Univariate analysis

Variable	Result (n=55)	
Sex	Female = 43 (78%)	Male = 12 (22%)
Age	56 ± 7.7 years (min 36 - max 70)	
CRP	<0.8 mg/dL (min<0.8 - max 12.8)	
Creatinine	0.55 mg/dL (min 0.2 - 2.3)	

Results of the C-Reactive Protein semiquantitative test are presented in Table 2. The level of C-Reactive Protein in 28 samples is in the normal value (<0.8 mg/dL) and 27 samples above normal. Based on the range of normal creatinine serum values obtained 4 patients with type 2 diabetes have values above the normal limit. Rank Spearman correlation statistical analysis is presented in table 3. There was a significant relationship between CRP and creatinine levels in T2DM patients with a weak positive correlation (r value=0.289).

Table 2. Results of the C-Reactive Protein semiquantitative test

	Level CRP (mg/dL)				
	<0.8	1.6	3.2	6.4	12.8
Frequency	28	6	8	10	3

Table 3. Correlation between C-Reactive Protein and Creatinine levels

		Creatinine Levels
CRP Levels	r	0.289
	p	0.033
	n	55

DISCUSSION

Table 1 show a higher percentage of women with diabetes mellitus. Women have a greater risk of developing diabetes mellitus, especially during postmenopause.[11,12] The derivation of estrogen in postmenopausal women catalyzes the development of insulin resistance and type 2 diabetes mellitus. Estrogen and progesterone hormone therapy increases the response of insulin receptors in postmenopausal.[13] Table 1 also shows the average age of respondents is 56 ± 7.7 years with the youngest and oldest age was 36 years old and 70 years old, respectively.

Aging can cause decreased organ function, including the endocrine system. The performance of the endocrine glands will decrease thereby reducing the production of hormones including estrogen and progesterone. Activated estrogen receptor (ER) and estradiol receptor (ER) genes cause metabolic processes. These genes work together in insulin sensitivity and increased transport of blood glucose into cells. Increased age will reduce the expression of these genes so thus decrease

insulin sensitivity which causes the pathophysiology of type 2 diabetes mellitus.[12]

There were 28 samples that had the CRP levels of <0.8 mg/dL. This result indicated that there was no inflammatory process. Basically C-Reactive Protein is normally produced at < 3 mg/L level or equivalent to 0.3 mg/dL. C-Reactive Protein is an acute-phase protein which level will rapidly increase as a sign of inflammation and is not influenced by age or sex.[14] C-Reactive Protein levels will usually increase from 5-50 mg/dL. The time needed to increase CRP levels is 6-8 hours after inflammation and reaches its peak after 48 hours.[14,15] The remaining 27 people were in an inflammatory condition. They were estimated to have experienced hyperglycemia for longer time, which can cause complications from type 2 diabetes mellitus.[16]

Increased C-Reactive Protein level begins with hyperglycemia which causes cells to not get a supply of glucose as a source of energy. This causes breakage in the mitochondria, so the Reactive Oxygen Species (ROS) production in cells increases. Increased reactive oxygen species causes macrovascular and microvascular damage.[3] This damage increases in diacylglycerol (DAG) synthesis which subsequently increases Protein Kinase-C (PKC) expression level in cells as well. The increase in PKC activation is followed by an increase in Nf-kB which functions as transcription factor for pro-inflammatory cytokines.[4] Several proinflammatory cytokines that can increase CRP levels are IL-6, IL-1β and TNF-α.[6]

Table 3 shows the correlation between C-Reactive Protein and creatinine levels is weak positive (r value=0.289). Serum hs-CRP was also associated with serum creatinine concentration in diabetic nephropathy, but no association was observed in type 2 diabetic patients without any complications.[17] In this study, we used type 2 diabetic patients without complications. The results of a weak relationship between CRP and creatinine are thought to indicate acute inflammation which signal kidney damage. This shows that early screening of CRP and creatinine in diabetic patients without complications can be used as biomarkers for early detection of diabetes nephropathy. C-reactive protein (CRP) is related with advanced diabetic nephropathy in patients with type-2 diabetes. However, role of CRP in type 2 diabetes remains unclear. CRP promoted renal fibrosis by a CD32b-Smad3-mTOR pathway because blocking mTOR signaling with rapamycin inhibited CRP-induced CTGF and collagen I expression CRP may induce CD32b- NF-κB signaling to facilitate renal inflammation.[18]

Creatinine is a product of muscle metabolism that is associated with type 2 diabetes mellitus.[19] Creatinine is an indicator of good kidney function due to its role in maintaining creatinine at normal levels. There were 23 samples who showed low creatinine levels below normal values. Low creatinine levels indicate reduced muscle cell mass. Muscle cells are storage areas for glucose in the form of glycogen.[19] In type 2 diabetes mellitus, insulin resistance condition is associated with the breakdown of glycogen in muscle cells as a substitute for energy sources in muscle cells. Otherwise, increased creatinine level is a marker of impaired kidney function. One of the organs that can be damaged in diabetic patients is the kidney.[8] We assumed that an increase in CRP in patients with type 2 diabetes without complications can trigger kidney cell damage which is characterized

by an increase in serum creatinine. The level CRP and creatinine serum are thought to be able to be used as monitoring parameters for type 2 diabetes without complications progress into complications.

The limitation of this study was limited sample size and possible effect from type 2 diabetes duration. Therefore, in future research, it is suggested to further classify the diabetes duration so increased CRP and creatinine levels can be analysed with time post type 2 diabetes diagnosis.

CONCLUSION

There was a significant relationship between CRP and creatinine levels in T2DM patients with weak positive correlation levels. Based on this we assumed the presence of inflammation which indicates damage to muscle cells and impaired kidney function in regulating creatinine levels in the serum of type 2 diabetes mellitus patients.

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ACTA BIOCHIMICA INDONESIANA

RESEARCH ARTICLE

CLONING AND EXPRESSION OF *Plasmodium falciparum* LACTATE DEHYDROGENASE (*Pf*LDH) IN *Escherichia coli* BL21(DE3)

FF Masduki [1,2]*, Y Hotimah[1], R Rani[1], A Mawardi[3], ERPF Ramandey[3], A Fibriani[4], S Suhandono[4]

[1]*Biochemistry Division, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Bandung, Indonesia*

[2]*Bioscience and Biotechnology Research Center, Institut Teknologi Bandung, Bandung, Indonesia*

[3]*Department of Biology, Faculty of Mathematics and Natural Sciences, Cendrawasih University, Jayapura, Indonesia*

[4]*School of Life Sciences and Technology, Institut Teknologi Bandung, Bandung, Indonesia*

***Corresponding author : f.masduki@chem.itb.ac.id**

ABSTRACT

Background: Immediate and accurate diagnosis of malaria is essential for effective control of this disease. Immunochromatographic based rapid diagnostic tests (RDTs) are economical, simple to perform, and provide results in a relative short time, can be useful to assist effective management of malaria. The commercially available malaria RDT in Indonesia is still imported. Therefore, an effort to produce malaria RDT independently is necessary. One of the biomarkers used in RDTs is *Plasmodium* lactate dehydrogenase pLDH. The production and accumulation of pLDH during asexual stage or blood-stage in all human infected malaria parasites can be used to indicate parasites viability, which is correlated with the number of parasites present in the plasma of infected patients.

Objective: The aim of this research is to produce recombinant PfLDH in *Escherichia coli* BL21(DE3).

Methods: *Pf*LDH gene was cloned into pET30a expression vector to obtain a 6.2 kbp recombinant plasmid pET30a-*Pf*LDH. *E. coli* BL21(DE3) was transformed with pET30a-*Pf*LDH using the heat shock method. Then, *E. coli* BL21(DE3)- pET30a-*Pf*LDH was cultured in LB broth containing 50 mg/mL kanamycin and was induced by 1mM IPTG at 37^{o}C.

Results: SDS-PAGE and Western Blot analysis showed that recombinant *Pf*LDH was expressed with molecular mass ~30 kDa.

Conclusion: Recombinant *Pf*LDH is expressed in *E. coli* BL21(DE3) and can be used in further research for producing r*Pf*LDH as a biomarker for malaria RDT development.

Keywords : Lactate dehydrogenase, Malaria Rapid Diagnostic Test (RDT), *Plasmodium falciparum*, Recombinant protein

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INTRODUCTION

Malaria is a life threatening infectious disease, especially in tropical and subtropical areas. According to the World Malaria Report 2019, there were 1,474,636 malaria cases reported in Indonesia in 2018.[1] Malaria is caused by the protozoan parasite from the genus *Plasmodium*. There are five species of *Plasmodium* which infect humans; *P. falciparum, P.vivax, P.malariae, P. ovale,* and *P. knowlesi*.[2]

The causative agent of malaria are transmitted from one infected host to another by the bite of female *Anopheles* mosquitoes. The symptoms of the disease include high fever followed by chills and rigors. In endemic area, people with high fever sometimes were given antimalarial drugs without proper diagnosis, which could lead to antimalarial resistance. To avoid this, WHO recommend that proper diagnosis to all malaria suspected patients should be given before administering drugs.[2]

To accommodate this need, it is necessary to develop techniques for malaria diagnosis, which is fast, economical and accurate. One of the diagnostic test that fit those requirements is Immunochromatographic based rapid diagnostic tests (RDTs). It can detect *Plasmodium* antigens in whole blood samples, is inexpensive, simple to perform, and provide results in a short time. A biomarker that can be used for RDT development is *Plasmodium* lactate dehydrogenase. Parasite lactate dehydrogenase (pLDH) is an enzyme involved in the terminal phase of the Embden–Meyerhof pathway (glycolysis) of the malaria parasite.[3] Production and accumulation of pLDH can be used to indicate parasite viability both *in vivo* and *in vitro*[4] Moreover, pLDH was one of the first plasmodial enzymes shown to be electrophoretically, immunologically and kinetically distinct from that of the host.[5]

Those characteristics of pLDH are suitable for biomarker. There are already several commercial malaria RDTs using pLDH, unfortunately all are imported. The aim of this research is to clone the gene encoding *Pf*LDH into the expression vector pET30a, as well as to express recombinant *Pf*LDH in *E. Coli* BL21(DE3). To confirm recombinant *Pf*LDH, SDS-PAGE and western blot are applied. This research is expected to contribute for malaria case management, surveillance, and elimination in Indonesian and as a first step to make local malaria Rapid Diagnostic Test.

MATERIAL AND METHODS

Isolation of *Plasmodium falciparum* Genomic DNA and Amplification of the *P. falciparum* LDH Gene

Genomic DNA was obtained from *P. falciparum* infected blood samples kindly provided by Balai Penelitian dan Pengembangan Kesehatan, Kementrian Kesehatan Jayapura, Indonesia (Ethical Clearance Nr 445/658/RSUD/2018). Genomic DNA was isolated from the blood samples according to the procedure described in Zymo Research Kit for Genomic DNA Isolation from Blood Samples. The *Pf*LDH gene was amplified by the Polymerase Chain Reaction (PCR) method using genomic DNA of *P. falciparum* as template. A pair of oligonucleotide primers were designed to amplify *Pf*LDH gene based on sequence of *P. falciparum 3D7 L-lactate dehydrogenase, Pf*3D7_1324900. The forward primer was *Pf*LDH-F (*Nco*I), ccatggATGGCACCAAAAGCAAAAA and reverse primer was *Pf*LDH-R (*Xho*I), ctcgagAGCTAATGCCTTCATTCTCT.

Recombinant Plasmid Construction

The amplified *Pf*LDH gene was cloned into pGEM-T easy resulted in recombinant plasmid, pGEM-T-*Pf*LDH and was sequenced by Macrogen Inc. (Korea). Thereafter, pGEM-T-*Pf*LDH and expression vector pET30a were digested with *Nco*I and *Xho*I. The digested *Pf*LDH fragment was then ligated to the digested pET-30a using T4-DNA Ligase to produce recombinant plasmid, designated pET30a-*Pf*LDH, where *Pf*LDH gene was tagged with 6xHis at C terminal site. The pET30a-*Pf*LDH was then sequenced by Macrogen Inc (Korea).

Transformation of *Escherichia coli* BL21 (DE3) and Recombinant *Pf*LDH Expression

Fresh *E. coli* BL21 (DE3) competent cells were made using $CaCl_2$ treatment method[6] and transformation of *E. coli* BL21(DE3) was performed by heat shock method. The screening of the resultant colonies was carried out by direct PCR colonies methods using insert specific primers. Expression of r*Pf*LDH was started by making overnight culture of positive colony. This culture was then transferred (1% v/v) into LB broth containing 50 g/mL kanamycin and incubated in 37˚C, 150 rpm for about 2 hours until OD_{600} reached 0.6. Bacterial culture was induced by adding 1mM IPTG and further incubated in 37˚C, 150 rpm for 3 hours. Centrifugation at 2,800 *g*, 4 °C was performed to harvest the culture.

Western Blot

The cell culture pellet was dissolved in sample buffer and boiled for 10 minutes before loading. SDS-PAGE was conducted as described by Schägger.[7] The gel was stained by Coomassie blue staining, except the gels for further analysis with Western blot. Protein from the SDS-PAGE gel was transferred to nitrocellulose membrane using the eBlot Protein Transfer System (GenScript, USA). The membrane was blocked by 5% (w/v) skimmed milk in TBST over night at room temperature, then washed three times with 10 mL TBST. The membrane was further incubated with Mouse 6x his antibody for one hour at room temperature, then washed three times with 10 mL TBST. The membrane was then incubated with anti-mouse-HRP-conjugates antibody for 1 hour at room temperature, washed three times with 10 mL TBST, then 5 mL ChromoSensor™ One solution TMB substrate (GenScript, USA) was added, shaken at low speed at room temperature until color formed.

RESULTS

The Recombinant Plasmid Construction and Transformation of *Escherichia coli* BL21 (DE3)

The *Pf*LDH gene was amplified as a 961 bp DNA fragment (**Figure 1**). As positive control, specific primers for amplification of IEBA175 RIII-V (1502 bp) were used to confirm that the genomic DNA used is still in good condition. The amplified 951 bp *Pf*LDH gene was first cloned into pGEMT easy cloning vector resulting in pGEMT-*Pf*LDH. Plasmid DNA was isolated and verified by sequencing. Sequence comparison with other available *Pf*LDH sequences in GenBank confirmed its identity (100%).

Thereafter, *Pf*LDH gene was inserted in-frame with initiator codon by cloning site *Nco*I and *Xho*I and C-terminal 6x His tag encoding sequence of bacterial expression vector pET30a under the control of inducible phage T7 promotor. The expression vector pET30a-*Pf*LDH is depicted in **Figure 2A**. The construct is predicted to encode recombinant *Pf*LDH with molecular weight of approximately 35.2 kDa.

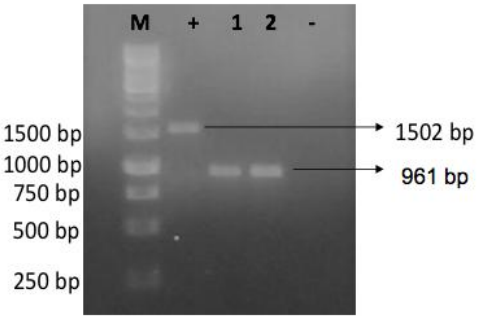

Figure 1. Amplification of *Plasmodium falciparum* Lactate Dehydrogenase (*Pf*LDH) was shown in lane 1 and 2 as DNA fragment (960 bp). Control positive (+) and control negative (-) was shown as well.

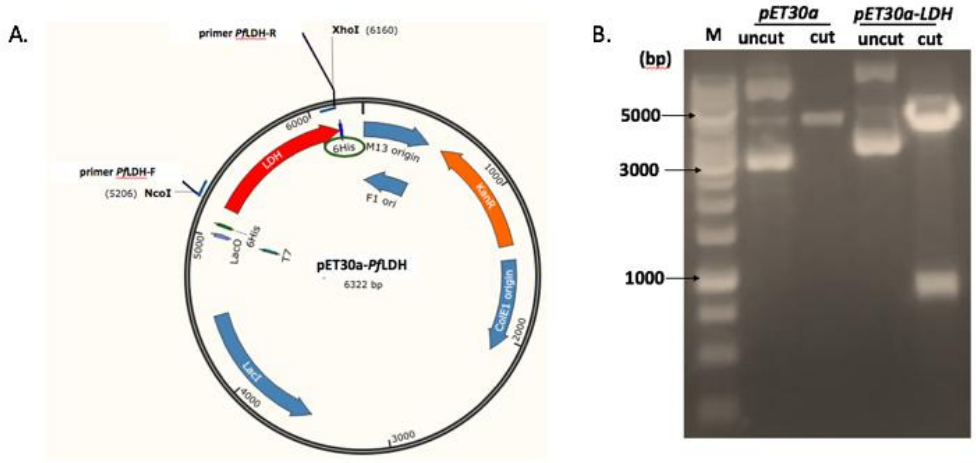

Figure 2. (A) Schematic representation of recombinant plasmid pET30a-*Pf*LDH construct carrying *Pf*LDH gene with 6x His tag at its C-terminus. The figure was created using Snapgene Software[11] (B) Restriction enzyme analysis of pET30a-*Pf*LDH recombinant plasmid using *Nco*I and *Xho*I enzymes with its uncut control.

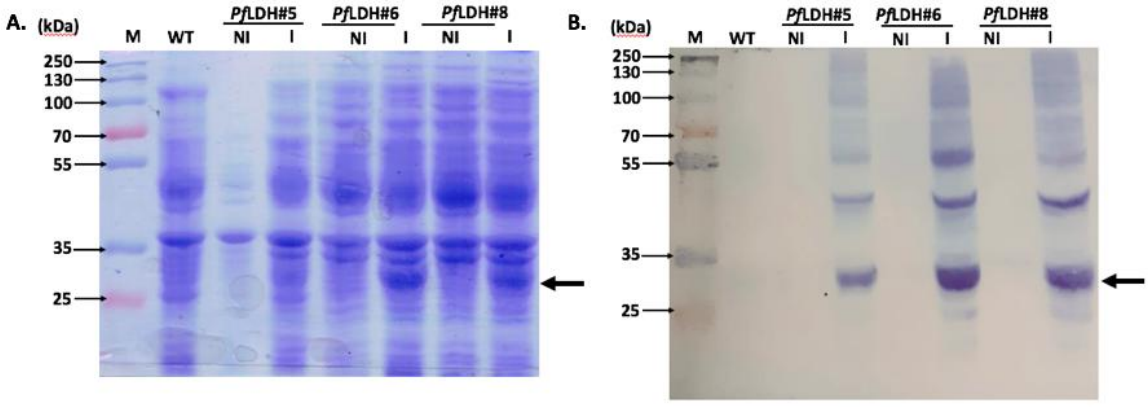

Figure 3. (A) SDS PAGE analysis of r*Pf*LDH expressed in *E. coli* BL21(DE3) and (B) Western Blot with anti-His antibodies. WT indicates wild type *E. coli* BL21(DE3) whole cell lysate as negative control, NI indicates uninduced whole cell lysate; and I indicates induced whole cell lysate. Tree positive colonies were analysed namely *Pf*LDH#5, *Pf*LDH#6 and *Pf*LDH#8. The arrows at the right indicate the position of r*Pf*LDH (34 kDa). The positions of molecular weight markers (M) are indicated on the left of the gel.

The recombinant protein was supposed to carry 6 additional histidin residues encoded by pET30a at its C-terminus. This vector was then transformed into the *E. coli* BL21(DE3) and transformants were selected in the presence of kanamycin. Ten randomly picked colonies were then confirmed using PCR. From the PCR positive colony, recombinant plasmid pET30a-*Pf*LDH were isolated and were verified using restriction enzyme analysis (**Figure 2B**).

Recombinant *Pf*LDH Expression in *Escherichia coli* BL21 (DE3)

Cultures of three positive clones,designated clone 5, 6 and 8, which were previously selected on the basis of PCR screening, were subjected to IPTG induction at 37 °C to identify clones which expressed the predicted 35.2 kDa r*Pf*LDH. SDS-PAGE analysis results shown in **Figure 3A** represent the induction profile of those clones. From the figure it is indicated that IPTG induction of *Pf*LDH gene expression was accompanied by the appearance of approximately a 33.4 kDa band. In contrast to induced transformed cells, untransformed cells (WT) cells failed to express the 33.4 kDa protein after induction with IPTG. As expected, uninduced transformed cells also failed to express the 33.4 kDa protein. To confirm that the 33.4 kDa protein is indeed *Pf*LDH, western blot with anti-His antibodies was performed. From the three clones identified, clone 6 showed the highest level of protein expression. This clone will be used for further research.

Western Blot

The molecular mass of r*Pf*LDH in crude extract and also the identity of r*Pf*LDH was confirmed by Western blot analysis using anti-His antibodies. Although unspecific binding occurred, there appeared a single band of protein around ~30 kDa in samples that were derived from positive clones 5, 9 and 17 (**Figure 3B**). The same band of protein did not appear in the sample that was derived from non-induced and WT.

DISCUSSION

*Pf*LDH is widely used as a biomarker for the identification of *P. falciparum* in Immunochromatography based Rapid Diagnostic Test.[8,9] In this research, recombinant *Pf*LDH was produced in three positive clones of *E. coli* BL21(DE3)- pET30a-*Pf*LDH.[5,6,8] In SDS-PAGE analysis, r*Pf*LDH is predicted as an approximately 30kDa protein (**Figure 3A**). This prediction is also supported by western blot analysis, where induced cells showed reactivity with anti-His antibodies at a protein fragment with molecular weight approximately 30kDa. Whereas, the estimated molecular weight of r*Pf*LDH is 35.4 kDa. Previous study[10] showed that there is possibilitiy the sequence starts at an internal methionine residue (19th residue of *Pf*LDH). r*Pf*LDH expressed from 19th residue will have a molecular weight of 33.4 kDa, which is detected by SDS-PAGE and Western Blot. From both analyses, it could be concluded that r*Pf*LDH produced in this study is in uncomplete/truncated form. However, the expressed protein still has His-tag for purification purpose.

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ACTA BIOCHIMICA INDONESIANA

RESEARCH ARTICLE

COMPUTATIONAL DESIGN OF ANCESTRAL AND CONSENSUS SEQUENCE OF APICAL MEMBRANE ANTIGEN 1 (AMA1) OF *Plasmodium spp.*

R Nurdiansyah[1*], RA Kemal[2]

[1]*Department of Bioinformatics, School of Life Sciences, Indonesia International Institute for Life Sciences, Jakarta, Indonesia*

[2]*Department of Medical Biology, Faculty of Medicine, University of Riau, Pekanbaru, Indonesia*

***Corresponding author : rizky.nurdiansyah@i3l.ac.id**

ABSTRACT

Background: It is important to design a malaria vaccine targeting all human malaria parasites as well as non-human primate parasites to eradicate malaria and prevent zoonotic malaria. Apical membrane antigen 1 (AMA1) protein is shared by human-infecting *Plasmodium* species. Ancestral sequence reconstruction (ASR) and consensus sequence construction on AMA1 might be able to overcome the antigenic distinction between those species.

Objective: We aimed to computationally design the ancestral and consensus sequence of *Plasmodium* AMA1 protein and analyze the sequences for its putative immunogenicity.

Methods: We utilized bioinformatics software to computationally design ancestral and consensus sequences of AMA1 protein. AMA1 protein sequences of human-infecting *Plasmodium* and non-human primate *Plasmodium* were retrieved from PlasmoDB. ASR was designed using MEGA X while consensus was inferred using UGENE. Phylogenetic tree consisting of existing *Plasmodium* sequences and the ancestral sequence was constructed using IQTREE webserver and visualized with FigTree.

Results: Phylogenetic analysis showed that *Plasmodium spp.* were divided into 2 major groups, *P. falciparum* (Clade F) and non-falciparum (Clade NF) thus three ancestral and consensus sequences were designed based on each clade and both clades at once. Reconstructed ancestral sequences were located as sister branch for naturally occurring strains. On the contrary, consensus sequences are located within the branch of corresponding naturally occurring strains. Sequence analysis showed the presence of CD8+ T cell epitope in all computationally-designed sequences.

Conclusion: Ancestral and consensus AMA1 sequences are potential for further studies as a malaria vaccine candidate.

Keywords : AMA1, Ancestral sequence reconstruction, Consensus sequence, Plasmodium, Vaccine

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INTRODUCTION

Malaria is a persistent disease transmitted by parasite *Plasmodium spp*.[1] WHO reported there were 219 million cases with 435 thousand mortalities in 2017.[2] One way to cure malaria infection is through artemisinin combination therapy (ACT) which attacks the parasite in the blood-stage.[3] Unfortunately, the cases of ACT resistance are becoming prominent every year. Some studies reported ACT resistance in the South East Asia region.[4,5] Even though still partially resistant, the parasite will eventually become fully resistant if left unchecked. Moreover, there are also reports in the cross-species transmission from non-human primates to humans. *P. knowlesi* was once known for its infectious nature to macaque, but now it actually can infect the human in the Southeast Asia Region.[6,7] The potential zoonosis of malaria could be caused by the human habitation and also the adaptive nature of the parasite and vector.[8,9]

Those reports indicated that the parasite is evolving to gain an edge in infecting humans. To prevent that, a novel method needs to be devised in creating preventive or curative measures for malaria. In this sense, an evolutionary biology approach could be an alternative way. One of the approaches is using the ancestral and consensus sequence. Ancestral sequence reconstruction (ASR) is a tool to infer the primordial sequence from the contemporary sequences and represents the common ancestor for those sequences.[10] While the consensus sequence looked for the residues with the highest frequency at a certain position after multiple sequence alignment (MSA) of the extant sequences. Those residues at a given position reflect the relative importance for the whole sequence, such as common function or domain.[11] Several studies used this approach to design vaccines for viruses.[12,13] For example, ancestral and consensus sequences of HIV-1 envelope protein can be utilized to recognize the broader natural variant spectrum.[14]

A previous study in looking for a new target candidate found several proteins were shared in the *Plasmodium* species, one of them is apical membrane antigen 1 (AMA1).[15] This protein was found in the human infecting ones, including the newly zoonotic *Plasmodium* species, *P. knowlesi*, and several non-human infecting ones. AMA1 is expressed in the form 83-kD precursor and then cleaved to create a 66-kDa as an integral membrane protein with an ectoplasmic domain, a transmembrane domain, and a C-terminal cytoplasmic domain.[16] Interestingly, this protein is also one of the prime candidates for the new malaria vaccine in several malaria species, such as *P. falciparum* and *P. vivax*.[16–20] This is due to its location on the surface of malaria and one of the crucial protein for the infection properties of the parasite to red blood cells.[18,21] Additionally, this 622 amino acid (AA) long protein is expressed on both the liver and blood-stage, make it suitable for both anti-infection and anti-disease vaccine.[22,23]

It is also reported that the AMA1 has high antigenic diversity due to its sequence polymorphism[24]; a longitudinal study comparing the data from Mali with the published sequences in the database found about 200 unique haplotypes with some key changes of the amino acid residue in the putative invasion machinery binding site.[25] This could pose a challenge in creating the vaccine, even though most of the published studies only focus on *P. falciparum*.[23–25]

Interestingly, a study reported that the multi-allele AMA1 vaccine could give broad coverage against the diversity of AMA1, highlighting the need for a vaccine with a broad coverage.[24] To this end, the broad coverage vaccine could be achieved by targeting the conserved region in the protein.[25]

Based on those arguments, this study is trying to utilize the ancestral and consensus sequence on AMA1 protein to determine the potential vaccine candidate for several *Plasmodium* species at once. This approach mainly uses the phylogenetic analysis of AMA1 proteins from several species. In the end, the result could be valuable information in supporting the creation of the universal malaria vaccine.

MATERIAL AND METHODS

Data mining

AMA1 protein sequences from eight Plasmodium species were retrieved from the PlasmoDB database (https://plasmodb.org/) based on the previous data mining analysis.[15] Five plasmodia were known to infect humans (*P. falciparum*, *P. vivax*, *P. knowlesi, P. ovale*, and *P. malariae*) and the rest could infect non-human primates (*P. coatneyi*, and *P. cynomolgi*). One species infect murine (*P. berghei*) and served as outgroup. From those eight species, a total of 24 protein sequences were retrieved from the database (Table 1).

Phylogenetic tree reconstruction

The phylogenetic tree reconstruction was done twice in this study. The first one was to establish the relationship between the retrieved AMA1 sequences and to help in inferring the ancestral and consensus sequences. The first phylogenetic tree was reconstructed based on Hall's protocol.[26] Multiple sequence alignment (MSA) was conducted using the MUSCLE algorithm[27] and then the model selection was conducted using the IQTREE server (http://iqtree.cibiv.univie.ac.at/)[28]. The maximum likelihood tree was reconstructed using the Jones, Taylor, and Thornton with gamma distribution (JTT+G) model based on the best model selector and 1000 bootstraps to check the tree robustness and validity. MEGA X software was used to reconstruct the first tree.[29] The second tree was made after the ancestral and consensus sequence of AMA1 was inferred. Different from the first one, the tree was made using the IQTREE server even though the model selection was using the same method as before.[28] JTTDCmut+F+G4 and 1000 bootstraps were used to reconstruct the second tree with the ancestral and consensus sequence. FIGTREE software was used to modify all of the trees for publication purposes.

Ancestral and Consensus sequence inference and analysis

The ancestral sequence of retrieved AMA1 was inferred using MEGA X based on the first phylogenetic tree and the default parameter from MEGA X.[29] After that, the ancestral sequence from the falciparum and non-falciparum were retrieved. Consensus sequences were inferred using the consensus function in the UGENE software with a strict 50% cutoff consensus.[30] The ancestral sequence and the consensus sequence for each of the clade and both clades were analyzed and retrieved to create the final tree. Ancestral and consensus sequences were aligned to find the conserved region.

Table 1. AMA1 Sequences retrieved from the PlasmoDB database

Sequence code	Accesion Number	Sequence code	Accesion Number
P. berghei ANKA	PBANKA_0915000	*P. falciparum_IT*	PfIT_110038000
P. knowlesi_strain_H	PKNH_0931500	*P. falciparum_KE01*	PfKE01_110038000
P. knowlesi Malayan Strain Pk1 A	PKNOH_S120150200	*P. falciparum_KH01*	PfKH01_110037800
P. vivax_P01	PVP01_0934200	*P. falciparum_KH02*	PfKH02_110038700
P. vivax_Sal-1	PVX_092275	*P. falciparum_ML01*	PfML01_110038300
P. falciparum_7G8	Pf7G8_110037300	*P. falciparum_SD01*	PfSD01_110036100
P. falciparum_CD01	PfCD01_110038900	*P. falciparum_SN01*	PfSN01_110036600
P. falciparum_Dd2	PfDd2_110036700	*P. falciparum_TG01*	PfTG01_110037900
P. falciparum_GA01	PfGA01_110037700	*P. malariae_UG01*	PmUG01_09042600
P. falciparum_GB4	PfGB4_110040000	*P. ovale_curtisi_GH01*	PocGH01_09039800
P. falciparum_GN01	PfGN01_110038000	*P. coatneyi_Hackeri*	PCOAH_00026700
P. falciparum_HB3	PfHB3_110036900	*P. cynomolgi_strain_M*	PcyM_0938200

The observed conserved region was analyzed for epitope presence available in the literature. Additionally, the sequences were analyzed using VaxiJen (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) for immuno-protective protein prediction with 0.5 thresholds.[31]

RESULTS

Phylogenetic trees

The first phylogenetic tree (Figure 1A) consisted of only natural sequences (retrieved from the PlasmoDB). It showed that the AMA1 sequences were clustered into the *P. falciparum* group (Clade F) and the non-falciparum one (Clade NF). P. berghei was used as the outgroup and therefore was not included in the ancestral and consensus inference (Figure 1A). The clustering served as the basis of the ancestral and consensus sequences inference. When ancestral and consensus sequences were included in phylogenetic tree construction, the same cluster pattern as observed (Figure 1B).

All of the ancestral sequences were located in the sister branch of the extant sequences, while the consensus sequences were located within the sequences. Interestingly, both of the ancestral and consensus sequences from every AMA1 clade were located in the middle of the phylogenetic tree, near the outgroup. The consensus sequence of all species resided in the falciparum cluster while the ancestral resided in the non-falciparum cluster (Figure 1B).

Ancestral and Consensus sequence epitope analysis

Ancestral and consensus sequences were analyzed for epitopes that have been previously characterized. Compared to CD8+ T cell epitopes TLDEMRHFY and NEVVVKEEY from *P. falciparum* AMA1, ancestral and consensus sequences have the 520NEVV(V/I)K(E/D)EY peptide (Figure 2)[23]. Analysis with PROVEAN (provean.jcvi.org) showed that the V524I and E526D substitutions were neutral.

Computationally designed sequences were also analyzed for residues required for binding of the invasion-inhibitory monoclonal antibody, mAb 4G2, to *P. falciparum* AMA1.[32]

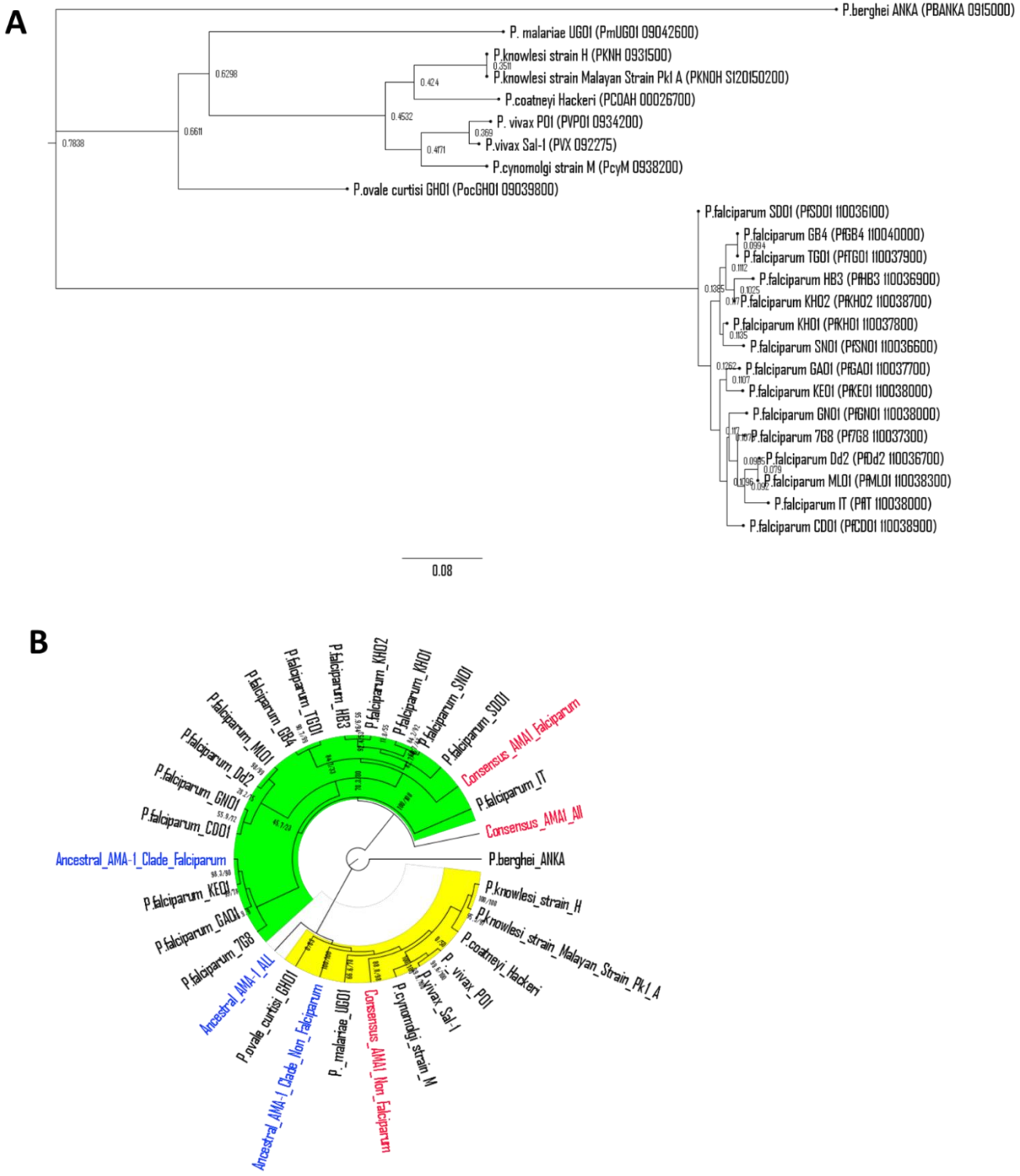

Figure 1. Phylogenetic tree of AMA1 sequences. A. Natural sequences. B. Natural sequences with its ancestral and consensus sequences. Red colored sequences: Consensus sequences. Blue colored sequences: Ancestral sequences.

Figure 2. The alignment of ancestral and consensus sequences showed a relatively conserved CD8+ epitope.

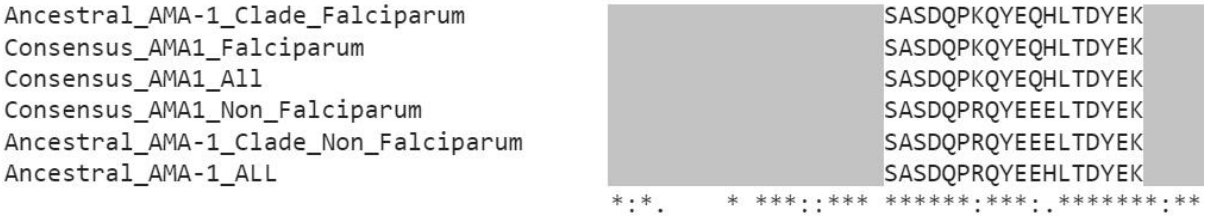

Figure 3. The alignment of ancestral and consensus sequences showed a relatively B-cell conserved epitope.

All of the sequences have conserved residues of Q352, F385, and D388. Consensus and ancestral sequences of the non-falciparum clade as well as the ancestral sequence for all clade had K351R and R389N substitutions. Analysis with PROVEAN showed that these substitutions were neutral. B-cell epitope characterized by *P. vivax* AMA1, SASDQPTQYEEEMTDYQK[33] was analyzed on the ancestral and consensus sequences. The epitope was present in all six sequences (Figure 3) with several substitutions. The epitope observed in the sequences was 345SASDQP(K/R)QYE(Q/E)(H/E)LTDY EK. PROVEAN analysis showed that the substitutions were neutral. Finally, analysis by VaxiJen showed that all computational sequences were considered as probable antigens with ancestral sequences that had a higher probability that consensus sequences (Table 2).

Table 2. AMA1 Sequences retrieved from the PlasmoDB database

Sequence	VaxiJen
	Antigen probability
Consensus Clade Falciparum	0.5798
Consensus Clade Non-Falciparum	0.5957
Consensus All Clades	0.5386
Ancestral Clade Falciparum	0.6402
Ancestral Clade Non-Falciparum	0.6566
Ancestral All Clades	0.6520

DISCUSSION

The phylogenetic tree construction positioned the consensus sequence of all species in the falciparum cluster.

The position of consensus sequence might due to the abundance of *P. falciparum* sequences in the database. However, even though the data mostly came from the *P. falciparum*, the ancestral sequence resides in the non-falciparum cluster. The ancestral AMA1 sequence might hint the evolutionary history of the *Plasmodium* species. This result is following the hypothesis of the evolution that the *Plasmodium* initially infected the non-human primates and then underwent zoonosis to humans.[8,34] The molecular pathway of this evolution was supported by an analysis of the ancestral sequence of *Plasmodium* RH5 protein.[34]

As one of the big three communicable diseases in the world, a lot of efforts have been done to combat malaria yet many challenges persist. The complexity of the *Plasmodium spp*. and its host-parasite interactions hinders the development in eradicating this parasite.[35] Interestingly, out of many proposed ideas, vaccine development has been considered to be the most feasible.[36–38] Some *Plasmodium* vaccine development has reached the trial version, even though the performance could be improved.[23,39,40] This, in turn, highlights the importance of the strong and long-lasting *Plasmodium* vaccine via the response of CD8+ T cells.[23] Besides the large size of the *Plasmodium* nuclear genome, the complex life cycle and the gene expression pattern of this species make it hard and challenging to do so.[41] In this regard, our target, AMA1 protein is expressed in both of life cycle during the human host period, the pre-erythrocytic which infects the liver and the blood-stage which infects the red blood cell, making it an interesting target in vaccine design.[42]

A putative AMA1 vaccine study detected CD8+ T cell response at epitopes TLDEMRHFY and NEVVVKEEY with the response frequency of 66.7% and 100%, respectively.[43,44] While we did not find the TLDEMRHY epitopes in any of computationally-designed sequences, our result using the human-infecting and non-human infecting species found the second CD8+ epitope, NEVV(V/I)K(E/D)EY, in domain III.[45] The presence of B-cell epitope in domain II[33] and recognition residues of mAb 4G2[32], as well as VaxiJen prediction for immunogenic protein, supported the hypothesis that all computationally-designed sequences to be immunogenic. However, this hypothesis needs to be further tested to develop a universal vaccine candidate against many human-infecting plasmodia.

CONCLUSION

This study provided the initial phase of the vaccine development of *Plasmodium spp*. based on the ancestral and consensus of AMA1 protein sequences. The clustering of the AMA1 sequences correlates with the current understanding of the host-parasite dynamics of *Plasmodium spp*. and it also revealed a relatively conserved epitope that could be recognized by the CD8+ cell, B-cell, and invasion-inhibitory antibody. Future studies should be focused on the potency of the conserved region as a vaccine candidate that could target many *Plasmodium* species at once.

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ACTA BIOCHIMICA INDONESIANA

RESEARCH ARTICLE

ROLE OF MALONDIALDEHYDE (MDA) IN PATIENTS WITH BREAST CANCER DISEASES

E Yerizel[1], N Astria[2], D Khambri[3]

[1]*Department of Biochemistry, Faculty of Medicine Andalas University, Padang, Indonesia*

[2]*Department of Midwifery, Faculty of Medicine Andalas University, Padang, Indonesia*

[3]*Department of Surgery, Faculty of Medicine Andalas University, dr. M.Djamil Hospital, Padang, Indonesia*

***Corresponding author :** nikiastria29@gmail.com

ABSTRACT

Background: Breast cancer is one of the most common cancers as well as one of the leading causes of cancer mortality in women worldwide. Cancer risk potentially continues to increase because of the many sources of exposure to carcinogenic chemical compounds. Carcinogenic compounds can contribute to free radical formation which might further interact and damage biomolecules such as lipids. Lipid peroxidation will increase malondialdehyde (MDA) levels, triggering gene mutations that leads to cancer.

Objective: The purpose of this research was to measure and compare MDA levels between breast cancer patients and control.

Methods: This research was observational research using a cross sectional comparative design of 30 breast cancer patients and 30 healthy controls. The place of this research is in Ropanasuri specialized surgery hospital and biochemical laboratory, Faculty of Medicine, Andalas University, Padang. This research was conducted from August to September 2019. The MDA was measured using spectrophotometer and independent T-test was done.

Results: The result of this research showed the mean MDA level of breast cancer patients was 3.98 ± 0.35 nmol/ml, higher than controls was 3.04 ± 0.36 nmol/ml with p value = 0.001.

Conclusion: There were significant differences in MDA levels among breast cancer patients and control in Ropanasuri specialized surgery hospital, Padang.

Keywords : Breast Cancer, Malondialdehyde

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INTRODUCTION

Breast cancer constitutes as the second leading cause of cancer deaths among women. Breast cancer development consists of several processes involving various cell types, making its prevention remains challenging in the world.[1] Cancer can be a fatal disease, making it among leading causes of death globally.[2] Breast tumors typically begin from the ductal hyperproliferation, and then develop into benign tumors or even metastatic carcinomas after constant stimulation by various carcinogenic factors. Tumor microenvironments such as the stromal influences or macrophages play vital roles in breast cancer initiation and progression. The mammary gland of rats are inducable into neoplasms only when the stroma was exposed to carcinogens, not the extracellular matrix or the epithelium. [3] Macrophages can generate a mutagenic inflammatory microenvironment, which can promote angiogenesis and enable cancer cells to escape immune rejection. [4] Different DNA methylation patterns have been observed between the normal and tumor-associated microenvironments, indicating that epigenetic modifications in the tumor microenvironment can promote the carcinogenesis.[5]

Based on the World Health Organization (WHO), cancer is the second leading cause of death worldwide. In 2018, there were 9,6 milions of death caused by cancer. Among one in six deaths caused by cancer, five were of the most common cancers including lung cancer, breast cancer, colorectal cancer, prostate cancer and skin cancer.[6] According to the data of cancer (Globocan) in 2012, from 1,7 million cases of breast cancer in women worldwide, 47% were in developed countries and 52% in developing countries.[7] Incidents of breast cancer differ, 39% were recorded in Asia, 29% in Europe, 15% in America, 8% in Africa and 1.1% in Australia. From these data it can be concluded that the Asian continent is the continent with the highest breast cancer incidence.[8]

Based on Globacan estimates, International Agency for Research on Cancer (IARC) in 2012, the incidence of cancer among the Indonesian women was 134 per 100,000 population with breast cancer has highest incidence by 40 per 100,000 followed by cervical cancer 17 per 100,000 women. The mortality rate caused by breast cancer is 16.6 deaths per 100,000 population. In 2013 in the Indonesian Ministry of Health (2015), cancer incidence increased from 12.7 million cases in 2008 to 14.2 million cases in 2012. Estimated number of breast cancer cases in West Sumatra in 2013 was 2,285 cases, making West Sumatra as one of the leading provinces with breast cancer casess.[9]

The scale of the problem about breast cancer can also be seen from the number of cases of breast cancer found in Ropanasuri Surgical Hospital in Padang, the number of breast cancer cases is still high. In 2017, there were as many as 163 women, in 2018 as many as 204 women, and from January to September 2019 as many as 148 women. Based on research conducted by Harahap on Andalas University, risk factors that cause breast cancer are genetic factors, previous cancer history, hyperplasia, ionizing radiation, age of menarche, age of menopause, age at first time, number of births, use of hormone therapy and oral contrast, and lifestyle factors including physical activity, diet, and alcohol consumption.[10]

The potential risk of cancer continues to increase because of the sources of exposure to carcinogenic chemical compounds. Carcinogens, if exposed to humans, can contribute to free radicals formation in the body. Free radicals interaction with biomolecules can trigger the formation of cancerous cells. Cancer cells experience abnormal growth and differ from normal cells due to changes in gene expression or mutagenesis which leads to an imbalance of cell proliferation and cell death. Cancer cells can attack other tissues through blood vessels and lymph vessels.[11]

Carcinogenic compounds can contribute to the formation of reactive oxygen species (ROS) in the body. This ROS can interact with biomolecules such as DNA, lipids and proteins. ROS attack reaction on lipids can cause damage to lipids in the cellular membrane, forming lipid peroxidation which yield to Malondialdehyde (MDA). The low molecular weight malondialdehyde can be produced from the free radical attack on polyunsaturated fatty acids. The lipid peroxidation constitutes the oxidative conversions of polyunsaturated fatty acids to MDA, the main sensitive parameter of lipid peroxidation.[12] Malondialdehyde is a sign of oxidative stress, especially in various clinical conditions related to the process of lipid peroxidation.[13] Malondialdehyde can be formed when hydroxyl free radicals such as ROS react with fatty acid components of cell membranes so that a chain reaction is known as fat peroxidation. The fat peroxidation will break the chain of fatty acids into toxic compounds and cause damage to cell membranes.[14]

The mechanism of MDA formation through lipid peroxidation begins with the removal of hydrogen atoms (H) from long-chain unsaturated lipid molecules by hydroxyl radical groups (OH), making radical lipids. Then these lipid radicals react with oxygen atoms (O_2) to form peroxyl radicals, which subsequently results in MDA (with more than three unsaturated bonds).[15]

Malondialdehyde is a natural product from lipid peroxidation capable of DNA interaction to form different adducts, including Malondialdehyde-1-deoxyguanosine (M1dG). Malondialdehyde-1-deoxyguanosine is mutagenic and triggers carcinogenesis.[16] Increased ROS levels in cancer cells are often regarded as adverse factors that cause genetic instability. In cancer cells there is an abnormal increase in ROS with high oxidative stress which makes the cancer cells to be more susceptible to further oxidative stress.[17] Based on the background above, the researcher was interested to measure and compare MDA levels between breast cancer patients and control.

MATERIAL AND METHODS

This research had been approved by the Ethics Commission of The Faculty of Medicine, Universitas Andalas (No.381/KEP/FK/2019). We conducted an observational study using cross-sectional comparative design to measure and compare MDA levels between breast cancer patients and healthy controls. This research was conducted at the Hospital for Specialized Surgery Ropanasuri Padang with subjects of 30 breast cancer patients who have not received chemotherapy and radiation and 30 healthy people as controls. Criteria for both groups were: aged <50 years old or premenopausal and did not have comorbidities (hypertension, diabetes, and obesity). Research subjects were interviewed and blood drawn (3 ml) and then taken to the biochemical laboratory of

the Andalas University Medical School Padang for analysis. MDA level was measured using Thiobarbituric acid and results were read using a spectrophotometer.

To measure MDA levels, the blood samples were let stand for 30 minutes in room temperature and centrifuged (2000 rpm in 15 minutes) to get serum, then prepare the tube by the following procedure: aquades, standard, add 2.5 ml of 5% TCA at each tube, mix using a vortex mixer then centrifuge for 15 minutes, at a speed of 3000 RPM, after which it is taken using a 1 ml pipet, put in a tube according to the label and add 1 ml each of Na Thiobarbituric Acid and mix it using a vortex mixer then heat it in boiling water bath for 30 minutes then cool and finally read the absorbance with a spectrophotometer at λ 550 nm.

The data were processed using the Statistical Package for Social Science (SPSS). Statistical tests utilized were based on the distribution of the obtained data. If the data were normally distributed the independent T-test, but if the data were not normally distributed after log10 transformation of data, Mann-Whitney test will be utilized.

RESULTS

There were 30 breast cancer patients aged 39.83 ± 6.58 and 30 control aged 34.80 ± 7.40 (Table 1). The normality test was carried out using the Shapiro Wilk test (Table 2) and it was found that the MDA level in breast cancer patients and healthy controls were normally distributed with p> 0.05, then continued with the independent T-test. MDA levels were determined in 30 patients with breast cancer and 30 healthy controls, which is considered as a control group. Based on statistical test (Table 3) there was a significant difference in MDA levels in breast cancer patients and healthy controls (p = 0.001). MDA serum levels were higher in breast cancer sufferers compared to control.

Table 1. The age of the breast cancer patient and healthy controls.

Characteristic	Breast cancer mean±SD	Healthy controls mean±SD
Age	39.83 ± 6.58	34.80 ± 7.40

Table 2. Normality test results for MDA levels

	Groups	Shapiro-Wilk	
		N	*P value*
Levels of MDA	Breast cancer	30	0.128
	Healthy controls	30	0.106

Table 3. MDA comparison between breast cancer patient and healthy control

Groups	N	Levels of MDA (nmol/ml) Mean±SD	*P value*
Breast cancer	30	3.98 ± 0.35	0.001
Healthy controls	30	3.04 ± 0.36	

DISCUSSION

According to the research find out that the age of the respondent average in the breast cancer fertile women age and premenopause are 39.83 + 6.58 years and healthy controls subject 34.80 + 7.40 years. Among various factors known to be the initiators of breast cancer, age is a trigger

factor for breast cancer. The incidence of breast cancer according to research conducted by Pane et al (2014) states that the age range of breast cancer sufferers aged 27-81 years and the average age of 48 years, there is a tendency for cases to be diagnosed at an earlier age due to the lifestyle of the respondents. The research conducted by Thangjam et al (2014) also found that out of 507 cases of breast cancer, 160 cases (31.56%) were under 40 years old and 347 (68.44%) were over 40 years.[18]

Similar research was also conducted by Rahmatya & Khambri among 46 people, the highest age of breast cancer sufferers in the Surgery Section of RSUP Dr. M. Djamil Padang in 2012 was found to be in the age range of 40-49 years with an average age of 46.87 years. The youngest was 32 years old while the oldest was 67 years old. These results were also consistent with research conducted by Azamris in Padang in 2006 which stated that the peak age of breast cancer sufferers at RSUP Dr. M. Djamil Padang among the ages of 40-50 years (34.3%) with an average age of 46.7 years.[19]

The Statistical test results showed that the mean MDA level in breast cancer patients was 3.98 + 0.35 nmol/ml while the average MDA level in healthy controls was 3.04 + 0.36 nmol/ml. The result of independent T-test obtained p-value = 0.001, therefore there was significant difference in MDA levels between breast cancer patients and healthy controls. Increased MDA levels in breast cancer sufferers might be due to induction of breast cancer cells to increase ROS that can induce oxidative stress followed by molecular damage and including lipid peroxidation.[20]

ROS level elevation, redox balance alteration, and redox signaling deregulation are common hallmarks of cancer progression and treatment resistance. ROS generation is mainly contributed by mitochondria during oxidative phosphorylation. Elevated ROS levels detected in cancer cells might due to several aspects, such as high metabolic activity, cellular signaling, peroxisomal activity, mitochondrial dysfunction, oncogene activation, and increased enzymatic activity of oxidases, cyclooxygenases, lipoxygenases, and thymidine phosphorylases. Intracellular homeostasis is maintained by developing an immense antioxidant system including catalase, superoxide dismutase, and glutathione peroxidase. Besides these enzymes, important antioxidant glutathione and transcription factor Nrf2 also contribute to balancing oxidative stress. ROS-mediated signaling pathways activate pro-oncogenic signaling which promotes cancer progression, angiogenesis, and survival. Additionaly, to maintain ROS homeostasis and evade cancer cell death, cancer cells increase antioxidant capacity level.[21]

One of the most produced lipid peroxidation aldehydes is MDA. It can react with proteins and DNA causing gene mutations that will trigger the formation of cancer cells besides increasing MDA levels as a marker of cancer cell development.[22] Increased MDA in breast cancer patients is associated with excessive ROS production and deficiency of antioxidant defenses. Excessive ROS production is triggered by exposure to chemical, biological and physical carcinogenic substances. A significant increase in MDA in cancer along with a decrease in antioxidants indicates the higher levels of oxidative stress and lower levels of antioxidant defenses. This event plays an important role in tumor development and the pathogenesis that

results from gene mutations caused by increased levels of MDA.[20]

A similar research conducted by Sahu et al showed the increase in MDA levels in breast cancer patients with an average of 5.8 + 3.2 nmol/ml and a control group of 1.9 + 0.28 nmol/ml with p-value = 0.01 ($p < 0.05$) so that there were statistically significant differences between the breast cancer group and the control group. MDA is a product of lipid peroxidation caused by an increase in ROS in the body, which can lead to the development of breast cancer cells.[23]

CONCLUSION

There was a significant difference in MDA levels in breast cancer patients and healthy controls. MDA level measurement is expected to be used as an indicator of breast cancer in women who are often exposed to carcinogenic substances as an effort to detect and prevent breast cancer in women.

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ACTA BIOCHIMICA INDONESIANA

RESEARCH ARTICLE

ALANINE AMINO TRANSFERASE (ALT) SPECIFIC ACTIVITIES IN LONG TERM SYSTEMIC HYPOXIC RAT BRAIN TISSUES

R Ramadhani[1], AR Prijanti[2,3*]

[1]*Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*

[2]*Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*

[3]*Center of Hypoxia and Oxidative Stress Studies, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia*

***Corresponding author : aniretno@gmail.com**

ABSTRACT

Background: Brain as a very aerobic organ is sensitive to hypoxia. Energy scarcities must be overcome by gluconeogenesis, which uses alanine or lactate as starting material. The reaction is catalyzed by alanine amino transaminase (ALAT or ALT), also known as glutamate pyruvate transaminase (GPT).

Objective: To investigate whether the specific activities of alanine aminotransferase (ALT) increased in hypoxic rat brain.

Methods: This experimental study used rats exposed to systemic normobaric hypoxia during 14 days. A group of 5 rats was sacrificed in days 1, 3, 7 and 14. The specific activities of ALT were analyzed in their brains using a reaction coupled with lactate dehydrogenase (LDH) activities.

Results: The ALT specific activities in rat brain were very low. There was no significant increase of specific activities during long term hypoxia ($p > 0.05$).

Conclusion: The rat brain ALT has no role in gluconeogenesis.

Keywords : ALT, Brain, Gluconeogenesis, Hypoxia

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INTRODUCTION

Oxidation is a very important reaction for life being, in order to extract the energy and produce useful metabolites. As an electron transfer reaction, oxidation can use various compound as an electron acceptor. However, oxygen (O_2) is used when cells need a big amount of energies, which occurs in aerobic metabolism. In this type of oxidation, energy is maximally released in oxidizing high energy nutrient substrates such as glucose, fatty acids or amino acids. For this objective, O_2 transport has to be assured can attain cells, which is not always the case. Otherwise, the cell will undergo a lack of O_2 condition or hypoxia. Hypoxia is a condition, which is characterized by insufficiency of O_2 relative to the need of organism, organ or even only at tissue level.[1]

Among the organs, brain is a very aerobic one and therefore very sensitive to hypoxia.[2] Moreover, brain is practically very dependent on glucose as energy source. Hence the glucose supply has to be assured all the time.[3] One of the common amino acid which is frequently used as a started material of gluconeogenesis is alanine. By one step transamination reaction, this amino acid is immediately converted to pyruvic acid, which is ready to be integrated into glucose. The reaction is catalyzed by alanine amino transaminase (ALAT or ALT), also known as glutamate pyruvate transaminase (GPT).

We are interested to know, whether total body hypoxia for a relatively long period would affect the brain metabolism, especially the conversion of amino acid into glucose precursor, which can be used in gluconeogenesis. For realizing our aims, we use a number of male Wistar rats, placed in a normobaric hypoxic chamber for two weeks, while water and raisin are given ad libitum. A number of rats were sacrificed periodically and specific activities of brain alanine aminotransferase (ALT) were measured accordingly.

MATERIAL AND METHODS

This was an experimental study carried out by placing rats in a normobaric hypoxia condition for 2 weeks. Twenty rats were placed in a closed hypoxic chamber, aerated with a gas mixture containing 90% N_2 and 10% O_2 and pressure 1 atmosphere directly from a gas tube. As control group, four rats were placed in usual laboratory condition. Both groups, control as well as experimental, feed with a standard diet and had access to water freely. The experimental group animals were sacrificed in 1, 3, 7 and 14 days of hypoxia treatment by decapitation. The brains were taken immediately, placed in chilled PBS pH 7.35 containing PMSF and frozen in a deep freezer (-80^{O}C) until the time of analysis. The protocol was reviewed and agreed by an Ethical Committee (376/PT02.FK/ETIK/2009).

Animals

Twenty-four male, young adult Sprague – Dawley rats, aged 8 – 12 weeks, were obtained from Center of Veterinary Research (BALITVET), Bogor, West Java, Indonesia. All animals were adapted in our laboratory condition for 2 weeks.

Chemicals

PBS (phosphate buffered saline) 0.1 M pH 7.4 was prepared according to the usual method. The needed chemicals were the crystals of NaH_2PO_4, Na_2HPO_4, and NaCl (Merck). Antiprotease phenylmethane sulfonyl fluoride (PMSF) was purchased from Sigma. ALT kit for alanine amino transaminase determination

was from Randox and contained buffer solution, substrate, coupling enzyme, and coenzymes. Bovine serum albumin (BSA, Sigma) was used for total protein assay.

Brain tissue extract preparation

Tissues were frozen and thawed 3 times by placing the specimen in deep freezer (-80^{O}C) and then in a water bath 37^{O}C. At the end of the third cycle, the tissues were homogenized by an automatic tissue grinder using a pestle. Then, homogenates were centrifuged at 5000 rpm for 10 minutes, to obtain supernatant liquid for further analysis.

Total protein measurement

The brain supernatant subjected to total protein analysis, which was measured by Warburg methods.[4] In principle, the absorbance of samples and protein standard solutions is measured at 280 nm.[5] A solution of BSA in PBS with concentration I mg/mL was used as a stock standard solution. A number of dilutions in PBS of the stock standard solution, ranges from 25 mg, 50 mg, 100 mg, 200 mg, 300 mg, 400 mg and 500 mg/mL respectively were used to construct a standard curve.

ALT assay

The enzyme activities assay was performed according to the guidance in the kit manual, which is based on lactate dehydrogenase (LDH) coupled method as described by Whitaker.[6] Briefly, ALT catalyzes the conversion of L-alanine to pyruvate in the presence of a-oxoglutarate. The pyruvate is reduced into lactate by NADH in the presence of LDH. The reagent mixture containing the powder of a-oxoglutarate, NADH, and L-alanine was dissolved in 100 mL buffer at the time of the assay. Then 100 mL of supernatant was pipetted into a cuvette placed in a spectrophotometer, followed by 1 mL of the reagent mixture. The difference of optical densities between first minute and fourth minutes a were read at 365 nm. As a blank, 100 mL of ddH_2O was used in the place of 100 mL of supernatant. As in protein determination, all measurements were done in Duplo. The ALT activities were calculated using the following formula:

U/L = 3235 x DA365/minute

Specific activities of ALT, i.e total activities of the enzyme/mg protein were calculated by dividing values of unit/mL with mg of total protein in the same samples.

Data treatment

All of the experimental data were analyzed statistically using IBM SPSS Statistics 20.0 for Windows. As usual, normality and homogeneity data will be examined. If it is normal and homogeneous, the analysis will be continued by Anova. If the result is significant, the analysis will be continued with a post hoc test. Otherwise, if the data is not normally distributed and/or not homogeneous, the data will be converted to logarithmic form and the normality and homogeneity will be examined. If both are still not normally distributed and not homogeneous, then the analysis will be performed non-parametrically.

RESULTS

Brain protein content

Total brain protein, expressed as mg of protein/brain weight, are presented in table 1. At a glance, it seems that there is a tendency of increase of brain protein contents, with the highest value in day 3. However, Anova statistical analysis shows

that the difference is not significant (p>0,05).

Table 1. Means of rat brain protein during 14 days of normobaric hypoxia the experiment (mg protein/g brain weight)

Group	Mean ± SD	P value*
Control normoxia group	5.407 ±1.406	
D1 normobaric hypoxia group	6.783±1.676	
D3 normobaric hypoxia group	6.972±1.728	P > 0.05
D7 normobaric hypoxia group	6.530±0.745	
D14 normobaric hypoxia group	6.550±1.837	

**Anova Test*

ALT specific activities

Specific activities of brain ALT are expressed as U/mg brain protein. Statistical analysis indicated that the distribution of data is not normal nor the data homogenous. Conversion of all data to log form give the same results. It was decided to analyze the data in the non-parametric way. Consequently, the data cannot be represented by mean and should be expressed by median. Accordingly, the standard deviation should be changed with minimum-maximum range. The results are presented in Table 2.

The specific activities of rat brain ALT were very low. There is an impression that ALT specific activities in brain increase when the duration of exposure prolonged. However, nonparametric statistical analysis indicated that the differences are not significant (p>0.05).

Tabel 2. Brain ALT specific activities (U/mg protein)

Group	Median (Min-Max)	P value*
Control normoxia group	0.0108 (0.0081-0.0129)	
D1 normobaric hypoxia group	0.0108 (0.0081-0.0722)	
D3 normobaric hypoxia group	0.0119 (0.0065-0.0739)	P > 0.05
D7 normobaric hypoxia group	0.0156 (0.0065-0.0388)	
D14 normobaric hypoxia group	0.0178 (0.0075-0.0431)	

**Kruskall Wallis Test*

DISCUSSION

Metabolically, the brain is the most active organ in the body. It needs a large amount of energy to maintain its functions and its integrity.[7] Hence, brain is a very aerobic organ, which consumed about 20% of total inspirated oxygen. Brain depends almost absolutely on glucose as fuel.[3] Glucose is completely oxidized into CO_2 and H_2O, and releases a large amount of energy are fixed as ATP. Hypoxia, therefore, has a severe consequence for the brain, which, if it can not be overcome in a short time, will be fatal for this organ. There are several ways for organism to overcome hypoxia conditions. The acute phase of hypoxia (first seconds and minute) is usually overcome by physiological mechanisms. The hypoxia itself has a direct action on blood vessels, it causes vasodilation on cerebral and heart muscle blood vessels, which increases tissue perfusion. Hypoxia regulates also blood vessels via chemoreceptors in carotid and aortic bodies, which exerts its influence through neural system.[8] After the acute period, organism survival in hypoxia relies

on gene expression dependent mechanism, which is mostly undertaken by Hypoxia-Inducible Factors (HIFs), a group of three different transcription factors regulating a number of proteins needed in order to face the chronic hypoxia.[9] In general, all proteins which are regulated by HIFs are essential for surviving in a conditioned lack of oxygen. The proteins which are regulated by HIF can be grouped into proteins control red blood cell production and vascular system, proteins control the energy metabolism, the protein control cell development and the proteins control homeostasis and extracellular matrix integrities.[10]

Our study is a part of a larger research on normobaric hypoxia, in which internal organs of the experimental rats, under chronic normobaric hypoxia, were investigated. It was reported that during the experiment, the animals underwent hypoxia.[11] Under this condition, the HIF-1 increased practically in every organ such as liver [12], kidney [13], stomach [14] and heart [15]. Hence, undoubtedly the brain of hypoxic rats undergoes hypoxia too. In the lack of oxygen, cell cannot oxidize glucose completely into water and carbon dioxide and will release lesser energy. To meet the need for energy on the same level, cell should take up higher number of glucose, which can lead to hypoglycemia. To maintain the glucose at the minimal tolerable level, organism will synthesize glucose from non-carbohydrate compounds, known as gluconeogenesis. For this objective, organism uses protein, after degraded into amino acids, as raw material. One of the common amino acid which is frequently used as a started material of gluconeogenesis is alanine. By one step transamination reaction, this amino acid is immediately converted to pyruvic acid, which is ready to be integrated into glucose. The reaction is catalyzed by alanine amino transaminase (ALAT or ALT), also known as glutamate pyruvate transaminase (GPT).

ALT is distributed in various organs, and depending on the animal, higher specific activities are found in heart, liver, and kidney for sea lion, harbor seal and elephant seal.[16] The authors did not report ALT in brain. It is not clear, whether the activities in brain are nil or simply the authors did not measure. However, ALT was found in very low concentrations in rat brain and kidney.[17] In their report, the number of ALT is not expressed in usual way as activities, but in the weight unit, which is reported as 50 ng enzymes/mg protein lysate. In our investigation, we found the activities of ALT in brain tissue homogenate. We also found that the activities were very low. As seen in Table 2, it seems that the brain ALT specific activities tended to increase during the experiment. However, nonparametric statistical analysis indicates that the increase is not significant ($p>0.05$).

Almost all glucogenic amino acids can be converted to glucose, except leucine and lysine. Alanine and glutamine have the most important role, both are mobilized immediately from blood soluble protein (albumin) and from skeletal muscle.[18] Among both amino acids, alanine is much more important, because this is the eminent amino acid transported from muscle to the liver during physical activities and also in calories deficiency.[19] The glucose alanine cycle, which is also known as the Cahill cycle, is also important to assure glucose homeostasis in health as well as in disease.[20] For catalyzing this reaction, the role of alanine aminotransferase is very crucial, because it converts directly the alanine into pyruvic acid, which can

directly be integrated into gluconeogenesis pathway to produce glucose. Liver, kidney and small intestine are known as organs capable to carry out gluconeogenesis. However, liver is always the main place for gluconeogenesis, as it can synthesize the glucose from alanine (Cahill cycle) or lactate (Cori cycle). Recently, it was also reported that astrocyte in brain is able to perform gluconeogenesis, a process that is crucial for the survival neuron cell in the hypoxia condition. However, astrocyte gluconeogenesis uses lactate instead of alanine.[21] From this point of view, it is considered that specific activities of ALT in brain are very low because brain can synthesize glucose from lactate, therefore the increase of its activity during the hypoxia period was not significant compared to normal oxygen living rats.

CONCLUSION

In conclusion, systemic hypoxia for a relatively long period affects various organs, which try to overcome the scarcity of energy by gluconeogenesis, itself can use alanine and/or lactate as starting substrates. If the alanine acts as starting compound, the involved cell needs ALT to convert the alanine to pyruvate. As the brain has very low ALT specific activities, it can be said that hypoxic brain depended on liver gluconeogenesis for the glucose supply and on the local gluconeogenesis which starts from lactate.

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