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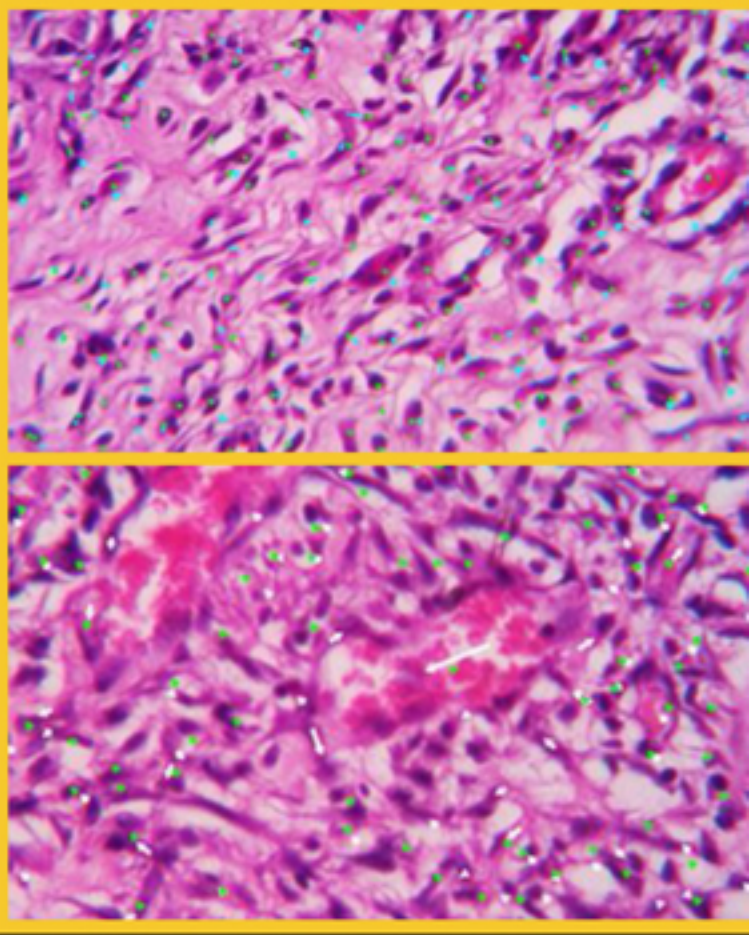

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Editorial

Quarter century of Medical Journal of Indonesia: Between national and international publications for building capacity building

Nafrialdi, Felix F. Widjaja

Medical Journal of Indonesia

The appearance of volume 26, number 4, 2017 of the Medical Journal of Indonesia (MJI) represents the closing of the publication year 2017. For the MJI, this year marks a quarter century of its age. It is indeed a quite long period for a scientific journal to become a highly reputable and widely known media for publication. Indeed, a significant leap has been done by the MJI along with its acceptation in the Scopus three years ago. It signifies that MJI's readability is widening worldwide. This success would not be accomplished without the quality and the regularity of MJI publication led by the previous editor-in-chief since MJI was born, Abdul Bari Saifuddin, Isnani A.S. Suryono, Nafrialdi, and now being led by Agus Rizal Hamid.

Being indexed in an international indexing company is an attracting factor for local researchers, and hopefully also for regional and international researchers to submit their manuscript to MJI. Hence, it is revealed that manuscript submission increased significantly. MJI is now flooded with manuscript submission, which should be seen as a good news. We hope that more quantity will bring better quality. Sufficient amount of manuscript enables us to increase the quality of the journal by selecting higher-quality papers to be published.

On the other hand, increased submission means a much bigger burden for editorial team and reviewers, unless more personnel are recruited. If not, this could eventually end with manuscript stagnation. On the other hand, it has been known that nowadays medical professionals "flooded" by articles. It is also our responsibility as a journal to assure the quality of published articles as an "ethical" knowledge.

Regulation of the Ministry of Research Technology and Higher Education number 44, 2015 stated that publication of research work is necessary for post-graduate student before a candidate is allowed to pass his/her final examination. For master degree program, the publication can be done in accredited journals with national or international reputation, while for a Ph.D. program, publication should be in a highly reputable international journal. This regulation gives a strong booster for the researchers to make publications and will bring important benefits to the national scope. International publication is one of the important points of measurement for university ranking. However, on the point of view of the local journal, this call brings some negative impact. If all the top quality researches done by Ph.D students are published in the high reputable international journal, then only second or third quality manuscripts left for local journals. Even though this local journals have been internationally indexed, their quality and citation are still very low, even some if it has no citation. We also can not solely assess a journal citation with Impact Factor from Thomson Reuters or CiteScore from Elsevier. Many articles in local journals are cited by other local journals that are still not indexed by Web of Science or Scopus which make them not calculated in Impact Factor or CiteScore. Ministry of Research Technology and Higher Education has introduced Sinta (Science and Technology Index) to observe the citation of accredited local journal by combining data from Scopus and Google Scholar. We hope that what meant by international publication not only limited to overseas journal, but will also cover internationally-indexed local journal with good citation locally and internationally.

It is interesting to note an article written by Prof. Idrus Affandi in a local journal/newspaper called "*Kabar Pendidikan"* edition January 6- 2018 urging to stop submitting to international publication. This author argued that obligation to publish in an international journal can be interpreted as a sort

of intellectual colonialism. Research findings from our country, he proposed, should be particularly readable for our community. This opinion seems right in certain aspects, but a complete cessation of international publication will lead to much more serious consequences that will end with the downgrading of national scientific quality. Participation at the international scientific community, including publication, is inevitable for research capacity building. Without international competition, our country will be left behind.

In conclusion, publishing in a journal with international reputation is a must to raise our position among the scientific community in the world, while national publication is also important to support the local journal increasing their quality.

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Basic Medical Research

Adipose derived stem cell conditioned medium effect on proliferation phase of wound healing in Sprague Dawley rat

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ABSTRAK

Latar belakang: *Kerusakan integritas kulit dapat menimbulkan kecacatan dan kematian. Kemajuan terapi luka pada saat ini telah merambah bidang sel punca dan* conditioned medium *sel punca asal jaringan adiposa (ADSC-CM). Masih sedikit hal yang diketahui tentang peranan ADSC-CM dalam mekanisme penyembuhan luka, pada proses angiogenesis, kuantitas kolagen, dan epitelisasi penyembuhan luka. Penelitian ini bertujuan menganalisis kadar* growth factors *(VEGF dan EGF) dalam ADSC-CM dan gambaran histologi ketiga proses tersebut (angiogenesis, densitas kolagen, dan epitelisasi) pascaluka insisi kulit hewan coba tikus Sprague Dawley.*

Metode: *Tiga puluh tikus dilukai pada bagian punggung (*full thickness wound*) dan diberi perlakukan berupa pembaluran ADSC-CM, medium kultur, medium basal, dan tanpa pembaluran. Tikus dikorbankan pada hari ke-3, 7, 14, 21 dan 28. Setelah dikorbankan, dilakukan pemeriksaan mikroskopik pada jaringan luka kulit. ADSC-CM yang dibalurkan diukur konsentrasi VEGF dan EGF dengan pemeriksaan ELISA.*

Hasil: *Terjadi peningkatan proses penyembuhan pada luka baluran ADSC-CM. Hal ini terlihat dari rasio epitelisasi dan penurunan jarak antarluka terbesar ditemukan pada luka pembaluran ADSC-CM.*

Kesimpulan: *Secara statistik tidak ada perbedaan bermakna antara kelompok luka yang dibalurkan ADSC-CM dan kelompok kontrol, tetapi secara klinis pembaluran ADSC-CM meningkatkan proses epitelisasi.*

ABSTRACT

Background: Disintegration of skin tissue can lead to disability and death. Recent studies on wound therapy applied stem cells and adipose derived stem cell conditioned medium (ADSC-CM) to improve wound healing. However, the role of ADSC-CM in wound healing mechanism in terms of angiogenesis, quantity of collagen, and epithelialization is not fully understood. Therefore, this study aimed to analyze the levels of growth factors (VEGF and EGF) in ADSC-CM and histological features of angiogenesis, epithelialization, and collagen density after skin incision in Sprague Dawley rats.

Methods: Thirty rats were injured at the back (full thickness wound) and treated topically with ADSC-CM, culture medium, basal medium, and without treatment. Mice were sacrificed on days 3, 7, 14, 21 and 28. After sacrificed, tissue samples were examined microscopically to assess angiogenesis, epithelialization, and collagen density. Concentrations of VEGF and EGF in ADSC-CM were measured by ELISA.

Results: Clinically, wound that was treated with ADSC-CM showed improvement in wound healing process. ADSC-CM treated wound showed the highest epithelialization ratio and the fastest wound closure.

Conclusion: There were no statistical significant differences between groups that were treated with ADSC-CM and not. However, topical ADSC-CM treated wound revealed a better clinical improvement in epithelialization.

Keywords: ADSC-CM, angiogenesis, collagen density, epithelialization, wound healing

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Skin is the largest organ in human body with a main function as a protector from external environment. The loss of skin integrity due to injury and illness may cost disability and mortality.[1] The prevalence of injuries nationwide in Indonesia by Riskesdas 2013 was 8.2%, and the major causes were falling down and traffic accident. There was an increase of injury prevalence in 2013 compared to 2007 according to Riskesdas. Abrasion was the third main injury that was experienced by the population.[2]

Wound healing is a series of stimulating and inhibiting processes such as cellular proliferation, differentiation, migration, and adhesion.[3] This series of events involves coordination of a variety of cells, growth factors, and cytokines.[3,4] Disruption of this coordination may inhibit wound healing.[4]

Wound healing is divided into 4 phases: hemostasis, inflammation, proliferation, and remodelling. In hemostasis phase, there are vasoconstriction and platelet aggregation in wound area. This phase is followed by inflammation that is marked by the presence of neutrophils, monocytes, and lymphocytes. Proliferation phase is an epithelialization of wound area, angiogenesis, and formation of collagen to replace fibrin that is formed at the stage of hemostasis. The last step of wound healing is remodelling and maturation of collagen in the healing tissue.[5]

A proper vascularization is very important to supply nutrients and oxygen to wound area in order to optimize wound healing. Angiogenesis is initiated by fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), transforming growth factor α (TGF-α), transforming growth factor β (TGF-β), and angiogenin. VEGF is a major mediator of angiogenesis to stimulate migration and proliferation of endotelial cells.[6] Epithelialization is a regeneration of epidermis that is stimulated by epidermal growth factor (EGF), PDGF, FGF-2 and TGF-β. The migration of epithelial cells will close the wound and followed by proliferation and differentiation of epithelial cells to form epidermis layer.[1] Collagen is a key component in providing strength to tissues. Collagen fibers are synthesized by fibroblasts and play a role in the wound closure.[7] The density of collagen can be assessed in histological specimens with special staining.

The application of stem cell culture derived conditioned medium as a therapy has been studied both in vitro and in vivo with excellent results. Therapy using conditioned medium is more promising than the stem cell itself due to the ease of production, packaging and distribution.[8] Previous *in vitro* study showed that adipose derived stem cell conditioned medium (ADSC-CM) could increase cell migration of keratinocytes, fibroblasts, and endothelial cells. Improving cell migration is expected to enhance angiogenesis, epithelialization, and fibroplasia through paracrine role of adipose derived stem cells (ADSC).[9,10]

The role of ADSC-CM in the in vivo process of angiogenesis, collagen density, and epithelialization are little known. Therefore, the aim of this study was to analyze the levels of growth factors (VEGF and EGF) in ADSC-CM and histological assessment of three processes (angiogenesis, epithelialization, and collagen density) after application of ADSC-CM to skin incision in Sprague Dawley rats.

METHODS

This was an experimental study using Sprague Dawley rats. Aged 8–12 weeks weighing 250–350 g. Animals were in good health during the research period and had bleeding time of less than 1 minute. Ethical clearance (No142/UN2.F1/ETIK/2015) was obtained from Ethical Committee of the Faculty of Medicine, Universitas Indonesia.

Thirty rats received four cuts in the back area (full thickness wound) 2 cm long with a depth of 5 mm. The four cuts of each rat were treated randomly with ADSC-CM (100%), complete culture medium, basal medium, and without treatment (control). The treatment was only done once after the rat skin injury. Therefore, there were four groups based on the treatment. Six rats were sacrificed at every time point on days 3, 7, 14, 21 and 28. After the rats

were sacrificed, scar tissue were examined microscopically.

ADSC-CM was obtained from Stem Cell Medical Technology Integrated Service Unit, Cipto Mangunkusumo Hospital, Faculty of Medicine, Universitas Indonesia. ADSC-CM was aliquoted and stored in a freezer at -20°C until it was used. ADSC-CM was obtained from passage-3 of ADSC culture that was cultured in complete medium, which contained α-MEM (basal medium) and 10% platelet rich plasma (PRP) in normoxia condition for three days. ADSC-CM derived from cells showed the characteristics of ADSC, i.e.: positive for CD 34 (low), CD 73 (high) and CD 90 (high), and could differentiate into three cell lineages: osteogenic, chondrogenic, and adipogenic lineages.

ADSC-CM contained various beneficial factors that were secreted by the mesenchymal stem cells, i.e. various kinds of growth factors such as EGF and VEGF, cytokines and membrane bound structures such as microvesicles and exosomes that contained various proteins, peptides, mRNA, small interfering RNA, etc. Complete medium did not contain cell secreted factors, and only contain basal medium and 10% PRP, while basal medium was a component of the complete medium.

Concentration of EGF and VEGF in ADSC-CM was examined by enzyme-linked immunosorbent assay (ELISA) using EGF (RAB0149) and VEGF (RAB0507) Sigma-Aldrich Elisa kit. All ELISA assays were conducted in four repetitions.

Tissue samples were processed into parafin blocks, cut, and then stained with HE and Masson's Trichome. The tissues were observed using a light microscope with a magnification of 40 and 400 times. Specimens were photographed by using Optilab advance plus camera and documented.

Histological evaluation was done in terms of angiogenesis, epithelialization, and collagen density. Angiogenesis was calculated on days 7, 14, 21 and 28 using a counting software on Optilab advance plus. Epithelialization was measured by epithelialization ratio and epithelialization length. Epithelialization ratio was the length of epithelial regeneration area divided by the length of the entire epithelialization area. Epithelialization length was the distances at the two edges of the wound epithelialization areas that were covered by epithelium. Epithelialization ratio and length were measured using measuring software of Optilab advance plus on days 3 and 7. The density of collagen was assessed in Masson's Trichome stained specimens using ImageJ software on day 14 and 28.

The data obtained were in the form of numbers. Data were computed into median and minimum-maximum or mean and standard deviation when appropriate, and presented in tables and bar diagram. Data were analyzed by analysis of variance (ANOVA) when they were appropriate for parametric test, or otherwise by non parametric test (Kruskal-Wallis) using Statistical Package for the Social Sciences (SPSS) version 22 to compare the four groups in terms of angiogenesis, epithelialization ratio and collagen density.

RESULTS

Thirty rats were injured at the beginning of the study. One rat died after wounded. The remaining 29 rats were analyzed until the end of the study. The concentrations (means ± standard deviations) of VEGF and EGF in ADSC-CM assessed by ELISA were 5052.7±0.3pg/mL and 0.2±0.1 pg/mL respectively.

Angiogenesis was analyzed on days 7, 14, 21 and 28. Figure 1 shows angiogenesis assessment on day 7 (A) and day 14 (B). Kruskal-Wallis analysis to compare the four groups in terms of angiogenesis showed that p values were higher than 0.05 (p day 7=0.547; p day 14=0.727; p day 21=0.796; p day 28=0.993) (Figure 1).

Epithelialization was assessed by calculating the length and the ratio of epithelialization on day 3 and day 7. Figure 2 shows assessment of epithelialization ratio. Our study found a wound with different behavior to other wounds i.e. a late epithelialization and wound gap widening compared to the previous day. Kruskal-Wallis test results in comparing the four groups on both parameters all showed that p values were higher than 0.05 (Figure 2).

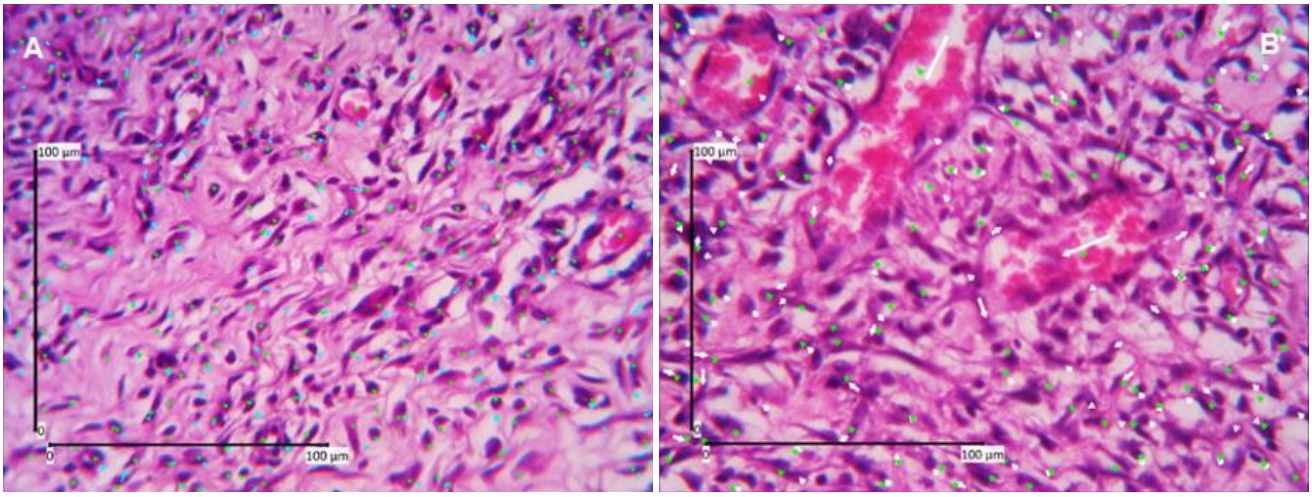

Figure 1. Angiogenesis assessment (HE staining, 400 times magnification). A) Day-7; B) Day-14, green dots show the calculation results of assessors one, blue and white dots show the calculation results of assessors two.

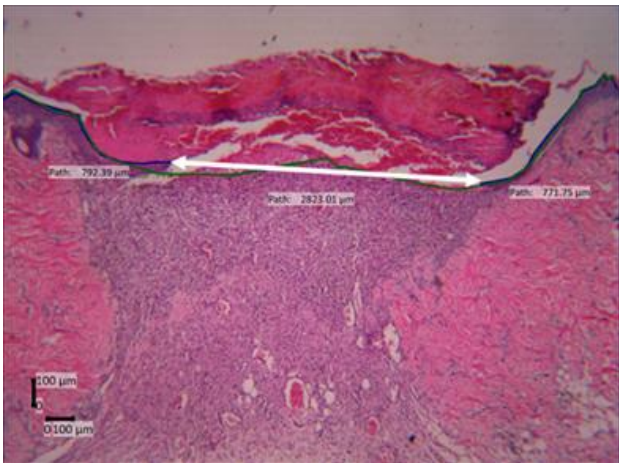

Figure 2. Epithelialization length and ratio assessment (day-7, HE staining, 40 times magnification). Green line=the length of the whole wound area, blue line=the length of epithelial regeneration area (epithelialization length), white double headed arrow=the distance of the two edges of remaining wound, epithelialization ratio=ratio of blue/greenline

Collagen density measurements were performed on day 14 and 28. Figure 3 shows collagen density assessment. Collagen density in skin wound preparations on day 14 and day 28 can be seen in Figure 4. Kruskal-Wallis test that compared the four groups in terms of collagen density showed p values that were higher than 0.05 (p day-14=0.707, p day-28=0.513). Figure 5 summarizes the results of angiogenesis, ratio, and length of epithelialization in the four groups.

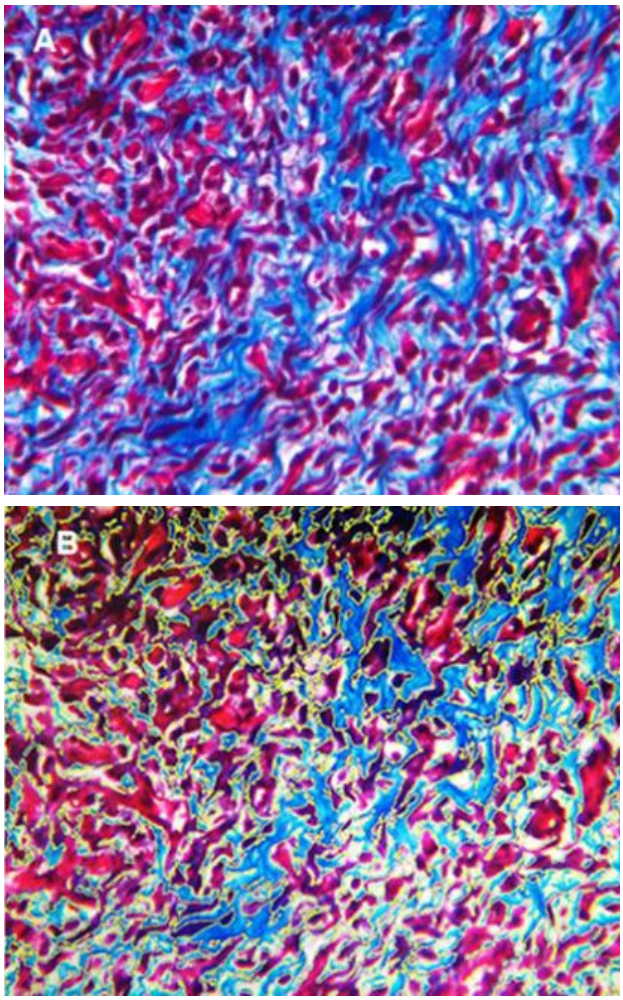

Figure 3. Collagen density assessment (Masson's Trichome staining, 400 times magnification day-14). A) original appearance; B) collagen density measurement, yellow line=demarcation of measured area that was analyzed by ImageJ

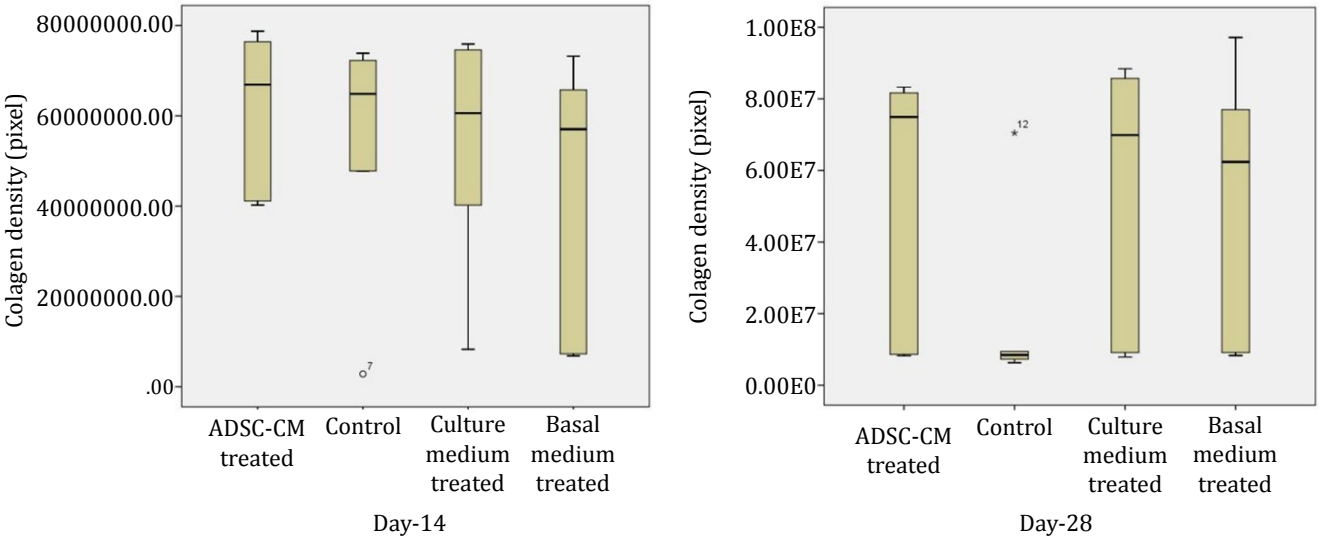

Figure 4. Collagen density in skin wound preparations on day-14 and day-28 in four groups

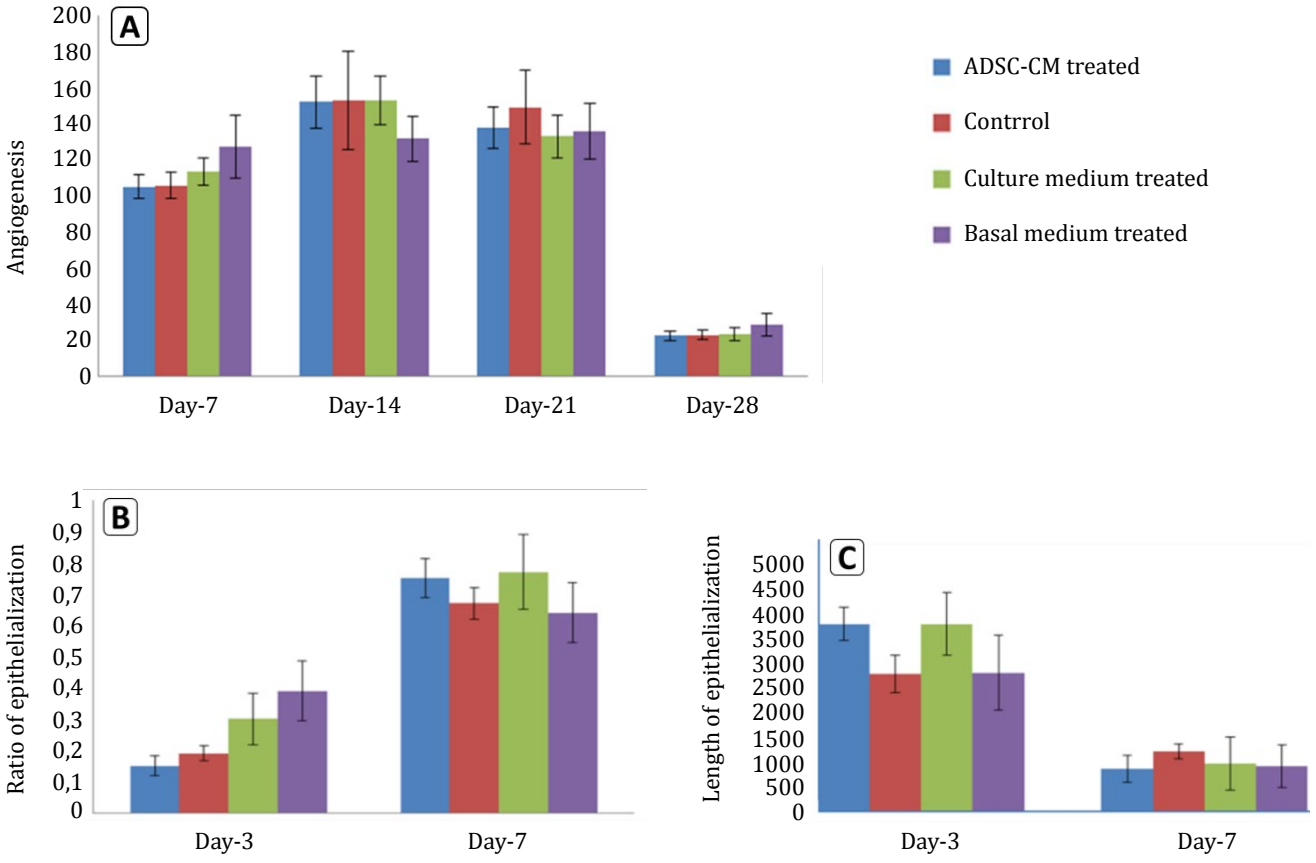

Figure 5. Angiogenesis, ratio and length of epithelialization in the four groups. A) angiogenesis; B) ratio of epithelialization; C) length of epithelialization

DISCUSSION

Our study showed that single application of ADSC-CM that was produced in normoxia, and three day incubation showed no statistical significant difference compared to control wounds during proliferation phase of wound healing although ADSC-CM caused faster epithelialization. A previous study revealed that mesenchymal stem

cells (MSCs) played a role in enhancing wound healing process either in intralesion injection or topical administration. Improvement of wound healing process was supposed to be a paracrine effect of MSCs rather than proliferation and differentiation of MSCs to replace damaged cells in wound tissue.[10] Moreover, recent studies revealed that conditioned medium from adipose stem cell cultures contained a number of growth factors and cytokines such as VEGF, EGF, PDGF, and TGF β.[8,11] VEGF is a specific mitogen for vascular endothelial cells. VEGF is a key component in the angiogenesis process of wound healing.[12,13] Therefore, in this study, topical administration of ADSC-CM was expected to enhance the process of angiogenesis in rat skin wound. Futhermore, epithelialization is one of the important processes of wound healing. Skin lesion will not heal if the epithelialization does not occur. This process is dominated by migration, proliferation, and differentiation into keratinosit.[14,15] Molecules that play a role in this process are EGF, FGF, and TGF-β. EGF is a key regulator of this process.[16]

The minimum effective concentration of VEGF to induce angiogenesis *in vivo* is 5000 pg/ mL.[17,18] The concentrations of VEGF and EGF in our study were measured by ELISA, and the mean concentration of VEGF and EGF in our ADSC-CM was 5052.7±0.3 pg/mL and 0.2±0.1 pg/mL. Compared to the result of another study by Kwon et al[17] a significant difference to our study was found as their VEGF concentration was 12.3±2.4 ng/mL. Our ADSC-CM was obtained from passage-3 ADSC culture in 10% PRP containing α-MEM and normoxia condition for three days, which can be regarded as cell culture waste product. In this study, we showed that cell culture waste product contained growth factors and can be used in various conditions that need growth factors for healing. Differences of VEGF and EGF concentrations in our conditioned medium from various other studies may be due to differences in the type and the origin of cells, cell culture method, culture condition and culture medium, which in our study were adipose tissue derived stem cells, two dimension culture, normoxia and 10% PRP containing α-MEM, respectively. Some studies used spheroid cell culture method in addition to either serum or insulin supplements in the basal medium hypoxic conditions, which can stimulate cells to secrete more growth factors.[11,19,20] Duration and serum-free culture medium can also influence the concentration of growth factors in ADSC-CM.[8]

In this study, we assessed angiogenesis, collagen density, ratio and the length of epithelialization in histological specimens. Statistical analysis showed no significant differences between ADSC-CM treated wounds and others, namely the control group, culture medium group, and basal medium group. As VEGF concentration in our conditioned medium met the minimal concentration that was required for angiogenesis, this non-significant differences might be due to the necessity to repeat the administration of ADSC-CM or systemic effects as the treatments and control were performed in the same rat, which was the limitation of our study. ADSC-CM contains cytokines and growth factors that have a short half-life, therefore it is advisable that repetitive ADSC-CM application is given.[8,21] Moreover, the non-difference between ADSC-CM and culture medium might be due to the presence of low level of growth factors from the 10% PRP.

In our study, angiogenesis was calculated based on blood vessel counting in specimens with routine HE staining. This staining poses difficulties in determining a blood vessel especially a small blood vessel when it is collapse. Moreover, most blood vessels that are found in angiogenesis are small blood vessels or buds. Therefore, the calculation might be lower than the reality. However, this limitation could be ignored as it is applied to the four groups.

In our study, clinically, ADSC-CM administration to incision wound showed the best result in parameter of epithelialization. ADSC-CM wound showed bigger ratio of epithelialization compared to control at day-7 and the fastest wound closure, which can be seen at the increase epithelialization ratio between day-3 and -7, and decrease in the length of epithelialization between day-3 and -7 (Figure 5). Our study found a wound with a late epithelialization and wound widening gap compared to the previous day. This fact might be due to stress factors in the rat. Stress factors can decrease wound healing process significantly.[5] Therefore, further studies are needed to analyze the role of these factors.

ADSC-CM effect on the density of collagen in vivo has not been reported yet. Collagen densities

in the four groups were all increased in day 28, compared to day 14. This fact showed that collagen has not entered remodeling phase yet. As the imbalance of collagen synthesis and reabsorption at the stage of remodeling can cause pathological scar.[6,22] A longer study in terms of collagen densities is recommended.

In vivo and *in vitro* process of wound healing are different. *In vivo* wound healing occurs in a very complex process.[23] Therefore, more studies are needed to analyze the role of ADSC-CM in wound healing, as ADSC-CM is a cocktail of various cytokines and growth factors that can affect various cellular and molecular activity of the wound healing process. We only analyzed EGF and VEGF levels, which might contribute to the healing process, but we did not address the other contents of ADCS-CM, and this is the limitation of our study.

In conclusion, though there was a clinical improvement in epithelialization of skin wound healing in Sprague Dawley rats, the improvement was not statistically significant, probably due to the single application of ADSC-CM.

Conflict of interest

Jeanne A. Pawitan is one of the editorial board members, but was not involved in the review or decision process of the article.

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Basic Medical Research

The preventive effect of *Mangifera foetida* L. leaf extract administered simultaneously to excess iron on markers of iron overload in Spraque-Dawley rats

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ABSTRAK

Latar belakang: *Saat ini belum terdapat obat untuk terapi pencegahan* iron overload *(IO) pada penderita talasemia. Penelitian in vitro maupun in vivo sebelumnya telah membuktikan ekstrak daun Mangifera foetida L. dapat menurunkan kadar besi pada tikus model IO. Penelitian ini dilakukan untuk membuktikan efektifitas ekstrak daun Mangifera foetida L. dalam mencegah terjadinya IO.*

Metode: *Tiga puluh ekor tikus Sprague-Dawley jantan dibagi ke dalam 5 kelompok: kontrol (tanpa perlakuan), IO, dan 3 kelompok terapi ekstrak daun Mangifera foetida L dengan dosis setara mangiferin 50, 100, and 200 mg per kg BB. Fe-dextran (15 mg) diberikan secara intraperitoneal dua kali seminggu selama 4 minggu untuk semua kelompok, kecuali kelompok kontrol (IO, DSM50, DSM100, dan DSM200). Sampel darah dan urin diambil sebelum dan setelah terapi 2 dan 4 minggu. Di akhir perlakuan, tikus dimatikan dan diambil organ limpa, hati, dan jantung. Parameter yang diukur adalah kadar feritin, kadar mangiferin, dan aktivitas SOD dalam plasma serta kadar besi dalam plasma, urin, dan limpa.*

Hasil: *Pemberian besi berlebih pada hewan model IO meningkatkan kadar Fe dan kadar feritin plasma dalam plasma sebesar 4,23 kali dan 6,9 kali dibanding normal. Ekstrak daun Mangifera foetida L. dosis 50 mg/kgBB menghasilkan kadar mangiferin dalam plasma tertinggi sebesar 212 ng mangiferin per mL dan terlihat, walau tidak signifikan, mencegah peningkatan kadar feritin dalam plasma dan IO di jaringan serta memproteksi stres oksidatif.*

Kesimpulan: *Ekstrak air daun Mangifera foetida L. kemungkinan dapat berguna untuk terapi preventif dan stres oksidatif pada pasien talasemia.*

ABSTRACT

Background: Recently, there is no agent available for the prevention of iron overload (IO) in thalassemia patients. Previous studies showed that *Mangifera foetida* L. leaf extracts reduced the levels of iron in IO *in vitro* and *in vivo* models. The present study aimed to determine the efficacy of *Mangifera foetida* L. leaf extract in the prevention of IO induced in rats.

Methods: Thirty male Sprague-Dawley rats were divided into 5 groups: control (untreated), IO, 3 treatment groups with leaf extract equivalent to 50, 100, and 200 mg of mangiferin per kg BW. Fe-dextran (15 mg) was administered intraperitoneally twice a week for 4 weeks to all groups except control (IO, DSM50, DSM100, and DSM200). Urine and blood samples were taken before and after treatments. After 4 weeks of treatment, rats were terminated, and samples of spleen, liver, and heart were taken. Ferritin and mangiferin levels and SOD activities were determined in plasma. Iron levels were measured in plasma, urine, and spleen.

Results: Experimental IO increased plasma Fe content 4.23 times and plasma ferritin levels 6.9 times vs normal. *Mangifera foetida* L. leaf extract DSM50 resulted in the highest blood levels of 212 ng mangiferin per mL and moderately, although not significant, prevented increased plasma ferritin levels and IO in organs and protected against oxidative stress.

Conclusion: Aqueous *Mangifera foetida* L. leaf extract may be useful to prevent IO and oxidative stress in thalassemia patients.

Keywords: Fe level, iron overload, Mangifera foetida L. leaf extract, mangiferin, SOD activity

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Iron overload (IO) is a serious chronic condition that develops when the body absorbs too much iron over years, and then excess iron is accumulated in organ tissues.[1] In thalassemia, iron overload can cause progressive organ injury before clinical symptoms develop.[1] Thalassemia is widely spread over the world covering the Mediterranean, Africa, Middle East, Burma, South East Asia, and Southern China.[2] In Indonesia, the prevalence of thalassemia is 0.1% with the highest numbers in Nanggroe Aceh Darussalam, South Sumatera, and Riau island.[3]

Thalassemia is an inherited disorder caused by mutations and/or deletions that decrease the synthesis of α- or β-globin chains. Impaired hemoglobin synthesis induces increased iron absorption from the gastrointestinal (GI) tract. In β-thalassemia major, severe anemia with massive erythroid hyperplasia in the bone marrow and heavy cachexia can occur.[4]

The supportive management of thalassemia includes blood transfusions to maintain hemoglobin levels of at least 9 to 10 g per deciliter (dL). These sporadic transfusions in thalassemia intermedia and regular transfusions in thalassemia major patients ameliorate anemia, improve the oxygen uptake into tissues, and reduce hematopoietic stimuli that can cause hepatosplenomegaly and bone deformities.[5]

Increased iron absorption from the GI tract of thalassemia patients and receiving (regular) blood transfusions leads to iron overload, frees iron in blood circulation, and accumulates iron in the visceral organs (e.g., liver, endocrine glands) and the heart. Excess iron in the systemic circulation can saturate transferrin (iron-binding protein in plasma). As a result, free iron will bind to other compounds with low molecular weight, e.g., citrate. Non transferrin-bound iron (NTBI) is easily absorbed by certain cells, including hepatocytes and cardiomyocytes. NTBI is also very sensitive to oxygen directing Fe(II) to the Fenton reaction which will generate superoxide anion radical and hydrogen peroxide.[1,2] Iron overload in myocardium and the iron-mediated generation of free radicals are associated with cardiovascular and other diseases and may eventually cause myocardial failure.[6]

Iron chelating agents were introduced in 1960 for the treatment of iron overload. The use of chelating agents aims to bind iron and prevent the formation of iron-mediated free radicals. Presently, deferoxamine, deferasirox, and deferiprone are used for iron chelating therapy, but all of them can cause severe unwanted side effects. Deferoxamine as the main standard drug has additional drawbacks such as lack of intestinal absorption and thus the inconvenience of parenteral application. Deferiprone and deferasirox can be administered orally, but they are too expensive for regular administration to thalassemia patients in Indonesia. Hence, it is necessary to search for novel chelating agents from domestic plants.[7]

One of the alternatives as chelating agent is mangiferin, which is contained *Mangifera* species, e.g., *Mangifera foetida* L. and some other medicinal plants. Many studies indicate that mangiferin exerts a wide pharmacological range, e.g., as antidiabetic, anti-HIV, anticancer, immunomodulatory, anti-oxidant, and iron chelating agent.[8–11] Its iron-chelating and free radical scavenging properties can prevent the early stages in the iron-induced formation of hydroxyl radicals.[12]

In rats with experimentally established iron overload, the therapeutic application of mangiferin and *Mangifera foetida* leaf extract reduced high plasma iron concentrations.[13] However, the prevention of iron overload *in vivo* has not yet been investigated. Therefore, the present work aimed to study the preventive effect of *Mangifera foetida* leaf extract towards the iron overload in Spraque-Dawley rats induced by administration of excess iron.

METHODS

Materials

Spraque-Dawley strain rats were purchased from the Indonesia National Agency of Drug and Food Control. *Mangifera foetida* L leaf powder was purchased from the Research Institute for Spices and Medicinal Plants (Bogor, Indonesia). Iron dextran (Fahrenheit, Indonesia), epinephrine (Sigma Aldrich, US), $NaHCO_3$ (Merck, Germany), NaEDTA (Merck, Germany), rat ferritin enzim-linked immunosorbent assay (ELISA) kit (Genway, US), TLC plate (Merck, Germany), mangiferin reference (Sigma Aldrich, US) were obtained at the highest purity available.

Mangifera foetida L. leaf extract

Mangifera foetida L. leaves were obtained from LIPI Cibinong in the form of analyzed dry powder. Dried powder was extracted using infundation at a temperature of 70°C for 15 minutes. This method refered to the research previously performed by Wahyuni.[13]

Mangifera foetida leaf extracts were prepared by infusion of 1 part of dried leaf powder in 5 parts of water at 70℃ for 15 minutes and processed to obtain a mangiferin-containing extract. Each infusion was carried out in triplicate. The solvent was evaporated with vacuum evaporator at 60℃ and 74 mBar pressure to obtain a viscous 'thick extract'.

Study design

This experimental study was conducted in Animal House and Pharmacology Laboratory from July 2014 until November 2014. Thirty male Spraque-Dawley rats were divided into five groups of 6 rats, each (normal, iron overload (IO), DSM50, DSM100, DSM200). The normal control group received no treatment. The animals in the iron overload group were administered iron dextran 15 mg intraperitoneally, twice a week for 4 weeks. Extract groups (DSM) were administered *Mangifera foetida* leaf extract equivalent to mangiferin 50, 100 or 200 mg/kg body weight (BW) daily for 4 weeks and iron dextran 15 mg intraperitoneally, twice a week for 4 weeks. After 4 weeks, all rats were killed. The blood of each rat was drawn. Moreover, spleen, heart, and liver were excised.[14,16] This study was approved by the Health Research Ethics Committee Faculty of Medicine, Universitas Indonesia, Cipto Mangunkusumo Hospital No 205/H2.F1/ETIK/2013.

Thin layer chromatography

Thin layer chromatography (TLC) was used to identify mangiferin content in the extract. TLC was performed using methanol-acetic acid-water (9:0.5:0.5) as mobile phase.[13] Mangiferin plasma concentration was analyzed using high performance liquid chromatography (HPLC) with a C18 column and PDA detector. The mobile phase was composed of methanol and 0.5% aqueous formic acid (30:70). The mobile phase was filtered through 0.22 µm membrane and degassed by ultrasound. The isocratic elution run time was 10 min at a flow rate of 1.0 mL per min.[14]

Further determinations

The measurements of iron were performed using atomic absorption spectroscopy (AAS). Samples were prepared by destruction of plasma, urine, and spleen sample with 1.0 mL nitric acid. Subsequently, the solution was heated on a hot plate until brown vapor disappeared. Furthermore, the solution was added to 5 mL of distilled water, and the iron content was measured.[15]

Superoxide dismutase (SOD) activity assay was carried out in accordance with Fridovic methods.[16] Ferritin plasma concentration was analyzed using Rat Ferritin-GWB-76152C ELISA kit Genway.

RESULTS

Mangiferin levels

Mangiferin was identified in the extract by thin layer chromatography (TLC). TLC of the ethanol fraction of the *Mangifera foetida* L. extract showed two spots with Rf 0.84 and 0.43 (Figure 1). Similar Rf values of the extract spot with the mangiferin standard Rf indicated mangiferin in the extract. Besides mangiferin, the extract also contained other compounds marked by other extract spots (Figure 1, lanes 2), i.e., alkaloids, flavonoids, saponins, and triterpenoid.

Furthermore, mangiferin was also proven by measuring mangiferin levels in the plasma of rats treated with *Mangifera foetida* leaf extract at week 4 of the experiment: DSM50 group 212 ng/mL; DSM100 group 116 ng/mL; DSM200 group 145 ng/mL (Figure 2). The peak obtained from *Mangifera foetida* leaf extract had the same retention time as the standard mangiferin indicating identical compounds.

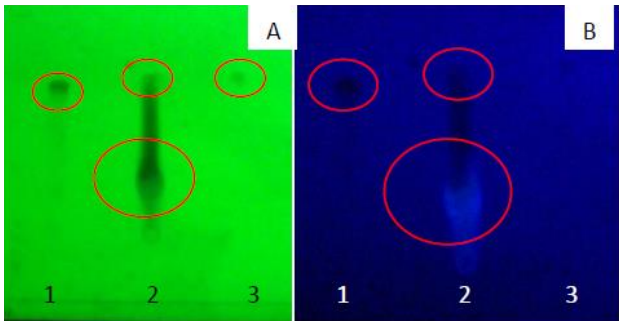


Figure 1. TLC of *Mangifera foetida* L. leaf extract detected at λ 254 nm (A) and λ 366 nm (B). Lane 1, mangiferin standard; lane 2, ethanol fraction; lane 3, hexane fraction

Plasma iron and ferritin

The experimental induction of IO in rats successfully increased plasma Fe content by 4.23 times and plasma ferritin levels by 6.9 times compared with the normal group (Figure 3 and 4).

The concomitant administration of *Mangifera foetida* L. leaf extract for 4 weeks lowered iron plasma levels in the DSM100 group and ferritin levels in the DSM50 group although statistically it was not significant (Figure 3 and 4).

Organ iron content

After four weeks of experimental iron overload and administration of *Mangifera foetida* L. leaf extracts, the organs (heart, liver, and spleen) in the iron overloaded groups had darker color compared to the normal group. The color change indicates the elevated levels of iron in the respective groups.

The spleen weight showed differences between the IO (1.26 g) and DSM50 (0.92 g) and DSM200 (0.83 g) although statistically it was not significant, indicating an influence of mangiferin on iron distribution into the spleen. Measurement of the total iron content in the spleen showed that DSM50 group had about 10% and DSM200 12% less total iron than the IO group (Figure 5).

SOD activity

After 4 weeks, SOD activity in plasma decreased along with the induction of iron overload (Figure 6). The administration of *Mangifera foetida* L. leaf extract equivalent to the lowest dose of 50 mg mangiferin (DSM 50) prevented this decrease in SOD activity, compared with the IO group. With higher doses of mangiferin, the protective effect got lost: in the DSM100 group, there was almost no protection vs. IO and in the DSM 200 group, SOD activity was even lower than in IO alone (Figure 6).

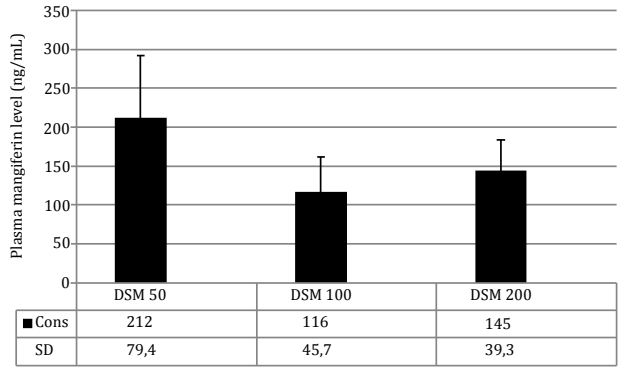


Figure 2. Mangiferin levels in plasma after 4 weeks of treatment with *Mangifera foetida* L. leaf extract

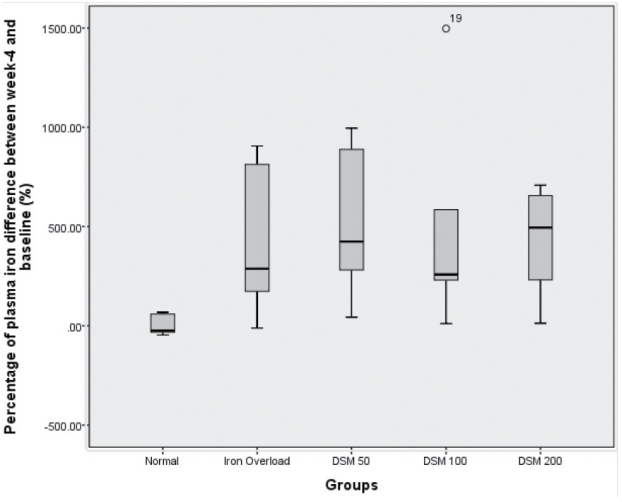


Figure 3. Percentage of plasma iron showing the differences between baseline and 4 weeks of iron administration

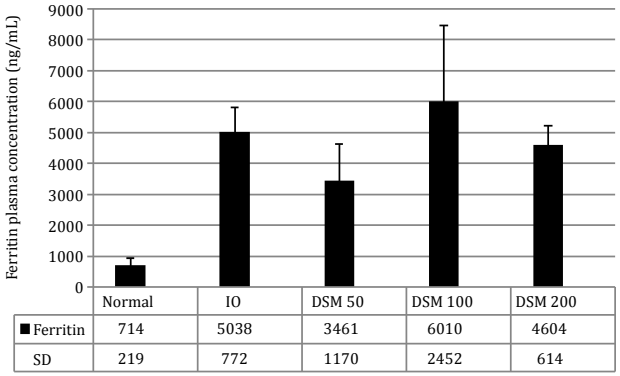


Figure 4. Ferritin plasma concentration

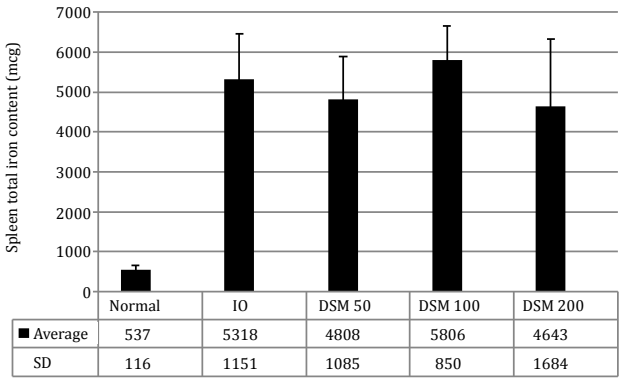
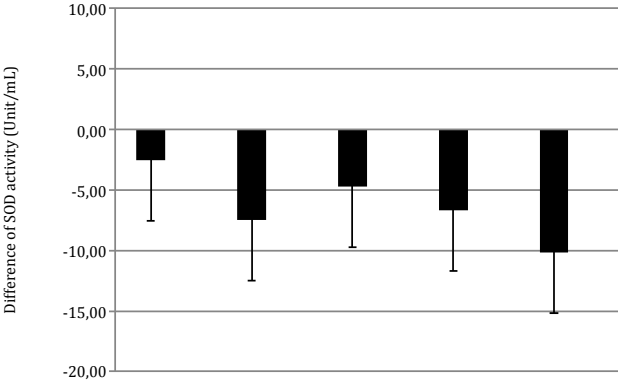


Figure 5. Spleen total iron content



	Normal	IO	DSM 50	DSM 100	DSM 200
■ Difference	-2,57	-7,46	-4,70	-6,71	-10,16
SD	5,54	10,86	17,00	8,38	6,84

Figure 6. SOD activity, difference from baseline after 4 weeks of treatment

DISCUSSION

Iron-overload is a common condition in thalassemia patients due to increased absorption from the gastrointestinal tract and additional blood transfusions.[1,5] High levels of iron concentration in thalassemia patients lead to increased free radical formation mediated by NTBI. The increase in free radicals is associated with final cardiovascular complications as a major cause of deaths in thalassemia patients.[7]

Mangiferin as a lead compound in *Mangifera foetida* L. leaf extracts has been known for its antioxidant activity. There are two antioxidant mechanisms of mangiferin against free radicals induced by iron; firstly, it prevents free radical generation by chelating iron and secondly, it scavenges free radicals.[17]

Water extract from leaves was used in this study because the glucoside of native mangiferin has better solubility in water than in ethanol. According to Kim et al,[18] mangiferin has the highest solubility in methanol followed by water and ethanol. We preferred water as extraction medium because it is less toxic than methanol, and aqueous extract is most easily made by the community.

Several studies have been conducted to assess the potential of *Mangifera foetida* L. leaf extract towards iron-overload. An ex-vivo study conducted by Pohan et al.[17] showed that mangiferin from *Mangifera foetida* L. leaf extract can bind to ferritin in blood plasma of patients with thalassemia. In another study, administration of *Mangifera foetida* L. leaf extract was equivalent to 75 mg/kg BW of mangiferin in experimentally iron-overloaded rats reduced iron plasma levels by 51% after one week of therapy and increased the elimination of 90% of Fe in urine.[13] The study showed that *Mangifera foetida* L. leaf extract had the pharmacological potential of reducing iron levels in iron-overloaded conditions. Therefore, the aim of the present work was to further document the ability of *Mangifera foetida* L. leaf extract in preventing elevation of iron levels in rats administered with excess iron.

The highest blood levels were obtained with DSM50. Increasing doses of the extract in the DSM100 and DSM200 groups did not lead to dose-response relationship in blood levels indicating non-linear pharmacokinetics of mangiferin.

Mangiferin has poor solubility and permeability in the gastrointestinal (GI) tract, with only 1.2% bioavailability.[19] Increasing doses of the extract are suspected of causing conglomerations, which make mangiferin absorption from the GI tract more difficult. In several studies, administration of more than 40 mg mangiferin resulted in non-linear pharmacokinetics of its absorption process.[19] From these results, only the administration of the extract equivalent to 50 mg of mangiferin per kg BW can be accounted for pharmacological effects.

In this study, the administration of *Mangifera foetida* L. leaf extract started when plasma iron conditions were still normal. Under normal conditions, almost all iron circulating in the blood binds to transferrin. Through the strong iron-transferrin, binding mangiferin cannot bind free iron and remove it from the body. Hence, the preventive effect will not be very strong and only moderate. Possibly, no significant pharmacological effects might be expected. That does not mean that mangiferin and the applied extract are ineffective because the iron-binding and iron-removing effect may increase with the increasing iron overload.

Plasma ferritin concentration is an indicator of iron-overload in humans and in this condition, therapy with chelating agents is initiated when ferritin levels are above 1,000 ng/mL or 10 times the normal ferritin concentration. At high ferritin concentrations above 3,000 ng/mL or 30 times the normal male ferritin levels, treatment with chelating agents decreased iron more effectively

than at lower ferritin concentrations.[20] This demonstrates the relationship between the plasma iron concentration and the effectiveness of chelating agents in binding it. In this study, plasma ferritin concentration in the IO group was 5,038 ng/mL or 7 times the concentration of the normal group. Thus, the concentration of ferritin in this study was intentionally lower than the concentration of ferritin in thalassemia patients when iron chelation therapy is started. Low plasma levels of iron and ferritin appear to be the cause of the relatively weak capability of *Mangifera foetida* leaf extracts to prevent the increase of iron in the body or to remove it through the urine (not shown).

The study design of Wahyuni[13] differed from this study. The former study examined the therapeutic effects of *Mangifera foetida* L. leaf extract towards experimentally established IO, whereas this study investigated whether this extract had also an effect in preventing experimentally induced IO in rats. The effect of *Mangifera foetida* L. leaf extract was much more expressed in the former[13] than in this preventive study design. In the condition of iron overload,[13] plasma transferrin was fully saturated, and high NTBI was formed that was easily bound by mangiferin. Our result raised an assumption that mangiferin from the extract was bound to plasma transferrin or ferritin and prevented the iron release from these proteins, thus diminishing the formation of NTBI. In a previous *ex-vivo*-study with thalassemia patients, *Mangifera foetida* leaf extract formed a complex with ferritin in serum.[21] Another assumption is that *Mangifera foetida* L. leaf extract increased the expression of iron binding proteins like transferrin and ferritin and inhibited the generation of NTBI via this mechanism.

SOD is an enzyme that converts superoxide anion radical into hydrogen peroxide in the body. Hydrogen peroxide is formed and then is converted into water and oxygen by catalase. Thus, SOD provides protection against superoxide free radicals by transforming them into compounds that are not toxic to the body. When the body is moderately exposed to free radicals, gene transcription of endogenous antioxidants is induced which leads to increased antioxidant activities that can detoxify free radicals.[22]

In this study, SOD activity in plasma decreased by the induction of iron overload. The ability of SOD in protecting tissues against free radicals is greatly affected by its concentration and the severity of the radical attack. SOD dose response curve to the protection capability is bell-shaped. In early stages of moderate oxidative stress, increased SOD expression and activity improved the protection against free radicals. Further increase of SOD concentration lowered the protection against free radicals. In studies conducted by Pardo-Andreu et al[12] and Wahyuni,[13] plasma SOD activity decreased with free iron confirming the results of this study.

Under massive experimental and pathological oxidative stress in skin, SOD activity decreased.[23] It was proposed that the increase in SOD activity develops superoxide radical dismutation to hydrogen peroxide, which in turn accumulates and attacks biomolecules, thus reducing the all-over protective effects of SOD.[24]

DSM50 showed a protective effect in this study, but this protective effect decreased with increasing doses of the extract. In the DSM200 group, there was significant decrease of the antioxidant activity indicating – under linear pharmacokinetic conditions - DSM overdose in this group leading to prooxidant activity. *Mangifera foetida* L. leaf extract contains mangiferin, flavonoids, and terpenoids. Mangiferin itself is known to have antioxidant and prooxidant activities. The mechanism of antioxidant mangiferin is iron binding, therefore inhibiting the formation of hydroxyl radicals and free radicals by scavenging with its phenolic catechol group. When mangiferin captures free radicals, its catechol group is oxidized transiently forming a semiquinone radical, which is potentially toxic to biomolecules.[25] This raises the suspicion that increasing doses of mangiferin may cause an increase in semiquinone radicals with increasing prooxidant effects rather than the antioxidant effects of lower doses. Moreover, other compounds in the extract such as flavonoids and terpenes may affect SOD activity in our experiments.

However, since higher doses of the applied extract created lower rather than higher blood levels of mangiferin, this non-linear pharmacokinetic effect should be accounted for the lower antioxidant effect of DSM200.

In conclusion, *Mangifera foetida* leaf extract equivalent to 50 mg mangiferin per kg BW exerts

moderately preventive effect on the parameters of experimentally induced iron overload in rats. Higher doses of mangiferin are not more effective because of non-linear pharmacokinetics. Thalassemia patients can be advised to use *Mangifera* leaves and fruit peels for preparing an aqueous infuse for daily drinks. Future research should better clarify the activities of *Mangifera foetida* L. leaf extract in the prevention of iron-overload. Some changes in study design, such as longer iron dextran induction period and/or extract administration can be expected to give more information about the capability of extract components, especially mangiferin to interact with iron-binding proteins, also using computer-simulated molecular docking.

Conflict of Interest

Melva Louisa and Hans-Joachim Freisleben are the editorial board members, but were not involved in the review or decision process of the article.

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Basic Medical Research

Bryophyllum pinnatum leaves ethanol extract inhibit maturation and promote apoptosis of systemic lupus erythematosus BALB/c mice B cells

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ABSTRAK

Latar belakang: *Sel B memegang peranan penting pada lupus eritematosus sistemik (LES). Menargetkan sel B sebagai terapi LES merupakan pendekatan yang paling memungkinkan. Bryophyllum pinnatum dengan kandungan metabolit sekundernya memiliki agen imunomodulator potensial. Studi ini menyelidiki efek potensial dari ekstrak etanol daun B. pinnatum dalam menurunkan persentase maturasi, meningkatkan persentase apoptosis, dan menurunkan ekspresi NF-κB p65 sel B mencit BALB/c dengan LES.*

Metode: *Studi in vitro dilakukan dengan melakukan kultur sel B dari limpa mencit BALB/c dengan LES yang diinduksi pristan. Sel B distimulasi oleh BAFF, LPS, IL-4, dan anti-CD40 menghasilkan CD19+>80%. Sel B dikultur dengan menambahkan stimulan tersebut dengan dan tanpa ekstrak etanol daun B. pinnatum (dosis 0; 0,02; 0,1; atau 0,5 µg/ml) selama 72 jam pada suhu 37°C. Persentase maturasi (CD19+CD38+) dan apoptosis (Annexin V+PI+) sel B diukur menggunakan* Flow cytometry*. Analisis lebih lanjut untuk mengetahui ekspresi faktor transkripsi dari maturasi dan apoptosis sel B, NF-κB p65, diukur menggunakan imunositokimia. Data yang terkumpul dianalisa menggunakan perangkat lunak SPSS versi 22.*

Hasil: *Uji* Flow cytometry *menunjukkan adanya penurunan signifikan terhadap persentase maturasi sel B pada semua dosis pemberian dan peningkatan signifikan terhadap persentase apoptosis sel B pada dosis 0,5 µg/ml. Hasil pemeriksaan imunositokimia menunjukkan penurunan signifikan terhadap ekspresi NF-κB p65 pada semua dosis. Persentase maturasi, apoptosis, dan ekspresi NF-κB p65 dari sel B memiliki korelasi satu sama lain.*

Kesimpulan: *Penelitian in vitro ini menunjukkan bahwa ekstrak etanol daun B. pinnatum menurunkan persentase maturasi, meningkatkan persentase apoptosis, dan menurunkan ekspresi NF-κB p65 sel B mencit BALB/c dengan LES secara signifikan.*

ABSTRACT

Background: B cells play a key role in systemic lupus erythematosus (SLE). Targeting B cells as SLE therapy is a plausible approach. This study investigated the potential effects of *Bryophyllum pinnatum* leaves with ethanol extract in decreasing percentages of maturation, increasing percentages of apoptosis, and decreasing NF-κB p65 expressions of SLE BALB/c mice B cells.

Methods: Culturing B cells from pristane induced SLE BALB/c mice's spleen will resulted in this *in vitro* study. B cells were activated by BAFF, LPS, IL-4, and anti-CD40 yielding CD19+ >80%. B cells were cultured by adding those stimulants with and without *B. pinnatum* leaves (0, 0.02, 0.1, or 0.5 µg/ml) for 72 hours at 37°C. Flow cytometry was performed to determine The Percentages of maturation (CD19+CD38+) and apoptosis (Annexin V+PI+) of B cells. Further analysis to determine the expressions of transcription factor of maturation and apoptosis of B cells, NF-κB p65, were performed using immunocytochemistry. Data were analyzed using SPSS version 22.

Results: Flow cytometry assay showed significant decrease in percentages of maturation of B cells in all doses and significant increase in percentage of apoptosis of B cells in dose 0.5 µg/ml. Immunocytochemistry results showed significant decrease expressions of NF-κB p65 in all doses. Percentages of maturation, apoptosis, and expressions of NF-κB p65 of B cells were significantly correlated.

Conclusion: This in vitro study revealed that *B. pinnatum* leaves with ethanol extract decreased the percentages of maturation, increased the percentage of apoptosis, and decreased NF-κB p65 expressions of SLE BALB/c mice B cells significantly.

Keywords: B cells, *Bryophyllum pinnatum*, pristane, SLE

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Among many autoimmune disease, SLE is one of the complex ones. It display heterogenity in clinical manifestation followed by several abnormalities such as: autoantibodies formation, immune complex deposition, and damages to the organs. B cells play a key role in SLE such as secreting autoantibodies by plasma cells, providing antigens as co-stimulant to T cells, inducing dendritic cell (DC) immunogenicly, producing proinflammatory cytokines and chemokines, and influencing the regulation of immunity and limphogeneity in SLE.[1] The plausible approach in therapy of SLE includes supression of autoantibodies production due to B cells maturation and proliferation, or B cell function modulation.[2]

To investigate the mechanisms of the disease, we need to understand how important are several SLE animal models to the mechanisms itself. One model, characterized by the development of several autoantibodies and clinical manifestation, is called pristane induced lupus model.[3] Intraperitoneal injection of pristane in BALB/c mice causes a SLE with high levels of immunoglobulin G (IgG), anti-double stranded DNA (anti-dsDNA), anti-Smith (anti-Sm), anti-RNP, anti-Su, anti-ribosomal P, and other lupus-related autoantibodies starting 12 weeks after injection.[4] This model may cause abnormalities in interferon production followed by defective clearance of apoptotic cells and over-active B-cell signalling. This is a clear impact of pristine-induced SLE BALB/c mice model.[5]

Due to earlier diagnosis and better treatment options of SLE, the prognosis has markedly improved in the last decades.[6] In the past decade, we have learn to fully understand the process behind pathogenesis of SLE, which led to a more effective therapeutic approach[7]. One of the examples of recent therapeutic approach is biologic agent for SLE. Biologic agent has generated substantial research interest for developing therapy of several autoimmune diseases.[8]

In 2011, biologic agent belimumab, a monoclonal antibody targeting human B cell activating factor (BAFF), was shown in randomized clinical trials to be efficacious in SLE and has now become the first approved targeted therapy for SLE.[8] Belimumab treatment is quite expensive. The total cost for the first year of treatment is around $28,000.[9] The study of BAFF and its clinical inhibition are gaining notable interests over the year. It was mainly due to the test of similar biologic agent in clinical trials.[10] Therefore, more effective drugs with an affordable monetary cost are urgently needed. In nature, there are many natural compounds which can be developed to treat SLE because their effects in modulate immunity.

B. pinnatum is a wild perennial succulent herb that is usually used as a traditional medicinal plant in tropical countries like Africa, Indonesia, and India. *B. pinnatum* is known by numerous vernacular names, such as life plant, love plant, and miracle leaf.[11] It has been used in anthroposophic medicine to treat various disorders caused by hyperactive conditions. Various secondary metabolites of *B. pinnatum*, especially flavonoid and bufadienolid, have been reported by several pharmacological studies as broad spectrum therapeutic potential such as immunomodulatory, cytotoxic and antitumor promoting activity, antiallergic, anti-inflammatory, antioxidant, analgesic, and antihypertensive. Other constituents of *B. pinnatum* include steroids, triterpenes, phenanthrenes, and some ubiquitous compounds.[12] Despite having broad spectrum therapeutic potential, *B. pinnatum*'s immunomodulatory activity in general and B cell apoptosis inducing property in particular have not been explored as yet. Therefore, this study evaluated whether *B. pinnatum* could decrease the percentages of maturation, increase the percentages of apoptosis, and decrease NF-κB p65 expressions of B cells furthermore prevent the development of pristane-induced lupus in SLE BALB/c mice B cells.

METHODS

Animals

This experimental and randomized post-test only controlled group design study was conducted in Malang, East Java, Indonesia between April and December 2016. Female BALB/c mice aged 6–8 weeks (25–35g) were purchased and certified from Pusat Veteriner Farma (Surabaya, East Java, Indonesia). All the BALB/c mice were housed at Pharmacology Laboratory of Universitas Brawijaya and acclimatized in the laboratory for 1 week prior to the experiments. The housing conditions were controlled, with a

room temperature and a diurnal 12-hour light/dark cycle. All experimental protocols described in this study were approved by the Health Research Ethics Committee, Faculty of Medicine, Universitas Brawijaya (Ethical clearance approval number: No. 328/EC/KEPK/05/2015).

Induction and treatment of pristane-induced SLE in mice

Female BALB/c mice were treated with single injection of pristane (Santa Cruz, California, USA) 0.5 ml intraperitoneally per mice. Spleen and serum were collected 16 weeks after the pristane injection. Clinical manifestations were also observed after 16 weeks of pristane injection. Consistent with our previous findings, treatment with single injection of pristane 0.5 ml intraperitoneally increased antinuclear antibodies (ANA) serum titers compared with control group as early 16 weeks (data not shown).

Plant material

B. pinnatum leaves were collected from Batu, East Java, Indonesia in August 2016. Leaves were identified and authenticated at Unit Pelaksana Teknis (UPT) Balai Materia Medica, Batu, East Java, Indonesia. They were air dried and grounded into fine powder. A sample specimen was deposited at the herbarium.

Extract preparation

Dried and powdered leaves (100 g) were exhaustively extracted with ethanol (900 ml) by static maceration at room temperature every 24 hours for 3 times. The ethanol extract was filtered and evaporated under a rotary vacuum evaporator at controlled temperature (70-75°C). This extract was filtered through whatman paper no. 42 (125 mm) and every 1 mg of stock solution was prepared in 1 ml (0.01%) of dimethyl sulfoxide (DMSO). The DMSO concentration was ignored because it was lower than 1%.

Cell preparation and culture

Mice were sacrificed by cervical dislocation 16 weeks after receiving the single pristane injection, and spleens were collected for analysis. Cell suspensions were prepared by homogenization in a tissue grinder. B cell enriched suspensions were obtained by stimulating suspensions with 100 ng/ml BAFF (Biolegend), 10 µg/ml anti-CD40 antibody (Biolegend), 20 µg/ml LPS from *Escherichia coli* (Sigma-Aldrich), and 50 ng/ml IL-4 (Biolegend). Our preliminary experiments demonstrated that this procedure yielded an enriched B-cell population >80% CD19+ cells as determined by flow cytometry analysis (data not shown). B cells enriched suspensions were cultured at a concentration of $5x10^5$ cells/ml in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibsco, Thermo Fisher Scientific) supplemented with 10% FBS and 100 U/mL penicillin-streptomycin (Sigma-Aldrich, USA), those stimulating factors mentioned above, with or without ethanol extract of *B. pinnatum* leaves (0, 0.02, 0.1, or 0.5 µg/ml) for 72 hours at 37°C without DMSO control.

CD19 and CD38

To determine the immunomodulatory activity of *B. pinnatum* leaves with ethanol extract against B cells maturation process *in vitro*, expressions of typical markers (CD19 and CD38) associated with the maturation status of B cells were measured. After 72 hours, cells were harvested for flow cytometry analysis of the percentages of CD19+CD38+ B cells. Cells were labeled with fluorescein isothiocyanate (FITC)-anti mouse CD19 (Biolegend) and phycoerythrin (PE) anti-mouse CD38 (Biolegend) following the protocol indicated by the manufacturer.

Apoptosis assay

Apoptosis of B cells were measured using flow cytometry staining with the FITC annexin V detection kit with propidium iodide (PI) (Biolegend). In detail, cells were stained for 20 minutes at room temperature in the dark with an annexin V. Afterwards, PI was added to the wells, and cells were further incubated for 5 minutes. After staining, cells were analyzed by flow cytometry to determine the percentages of apoptotic cells (Annexin V+PI+).

Detection NF-κB p65 expressions by immunocytochemistry

To understand the molecular mechanisms of B cells depletion, the expressions of protein controller of maturation and apoptosis of B cells, NF-κB p65, were examined using immunocytochemistry labeled with NF-κB p65. Immunocytochemistry was performed by using the streptavidin-biotin/indirect immunoperoxidase method.

Thin smears were fixed in poly-L-lysin coated slides by methanol for 30 minutes. They were washed in FBS 3 times for 5 minutes, and endogenous peroxidase was blocked using 3% hydrogen peroxide in methanol for 20 minutes at room temperature. Smears were washed in FBS 3 times for 5 minutes. Unspecific proteins were blocked using blocking buffer (3% FBS and 0.25% Triton X-100) for 6 minutes at room temperature. The primary antibody (NF-κB p65, Santa Cruz) was applied at the desired dilution, and slides were incubated overnight at 4°C in a moist chamber. They were washed 3 times with FBS and further incubated with the secondary antibody (Biotin conjugate) for 60 minutes, washed in FBS, and incubated with horseradish peroxidase for 40 minutes. By washing the slides again with FBS three times, and rinsing it with distilling water, can resulted in the development of the color 3–3' diaminobenzidine and hydrogen peroxide. Hematoxylin can be used afterwards to counterstain the smears. The final output, the brown reaction product (NF-κB p65), then would be mounted and observed under the light microscope. Total numbers of apoptotic cells were determined by calculating the number of brown color cells in 10 fields of view then divided by 10.

Statistical Analysis

The percentages of CD19+CD38+, Annexin V+PI+, and NF-κB p65 expressions of B cells were tested for normality using the Kolmogorov Smirnov test and for homogeneity variances prior to further statistical analysis. The data were normally distributed and were expressed as means ± standard error of mean (SEM). Significant differences among groups were analyzed by one way ANOVA followed by Tukey's post-test for multiple comparisons using SPSS software, version 22 (IBM Corp., Armonk, NY, USA). Correlations between the percentages of CD19+CD38+, Annexin V+PI+, and NF-κB p65 expressions of B cells were examined by Pearson's correlation coefficient. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Pristane-induced SLE BALB/c mice model

Clinical evidence of inflammation of joints, gait changes, alopecia, and ascites occurred 16 weeks after pristane injection in BALB/c mice (Figure 1). The first sign of joint inflammation was seen at 12 weeks after pristane injection (Figure 1A).

B. pinnatum leaves with ethanol extract inhibited maturation of pristane-induced SLE BALB/c mice B cells

This study showed that treatment with 0, 0.02, 0.1, or 0.5 µg/ml *B. pinnatum* leaves with

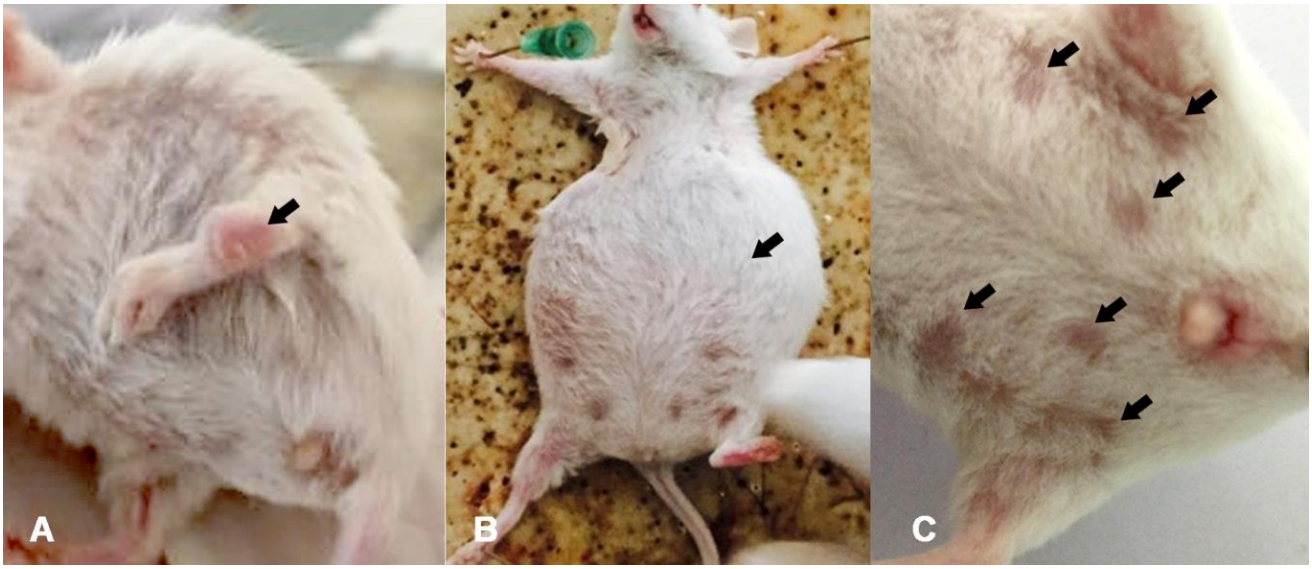

Figure 1. SLE clinical manifestations after 16 weeks of pristane injection. A) Joint inflammation was seen at 12 weeks after pristane injection (black arrow); B) Production of abdominal fluid or ascites (black arrow); C) The most frequent signs were alopecia (black arrows)

ethanol extract inhibited maturation rates of 6.64±0.14%, 5.90±0.46%, 5.54±0.31%, and 4.89±0.37% (Figure 2), respectively. $CD19^{+}CD38^{+}$ B cells percentages were significantly decreased compared to the untreated group (p=0.006, p=0, and p=0, respectively) (Figure 3A).

B. pinnatum leaves with ethanol extract promoted apoptosis of pristane-induced SLE BALB/c mice B cells

This study showed that treatment with 0, 0.02, 0.1, or 0.5 µg/ml *B. pinnatum* leaves with ethanol extract promoted apoptotic rates of 35.80±1.54%, 38.41±5.19%, 40.28±2.51%, and 44.01±2.60% (Figure 2), respectively. B cells treated with 0.5 µg/ml *B. pinnatum* leaves with ethanol extract significantly increased the percentage of apoptosis compared to the untreated group (p=0.002). However, lower dose treatment of 0.02 and 0,1 µg/ml *B. pinnatum leaves* with ethanol extract showed insignificant effect on increasing the percentage of apoptosis compared to the untreated group (p=0.520 and p=0.114, respectively) (Figure 3B).

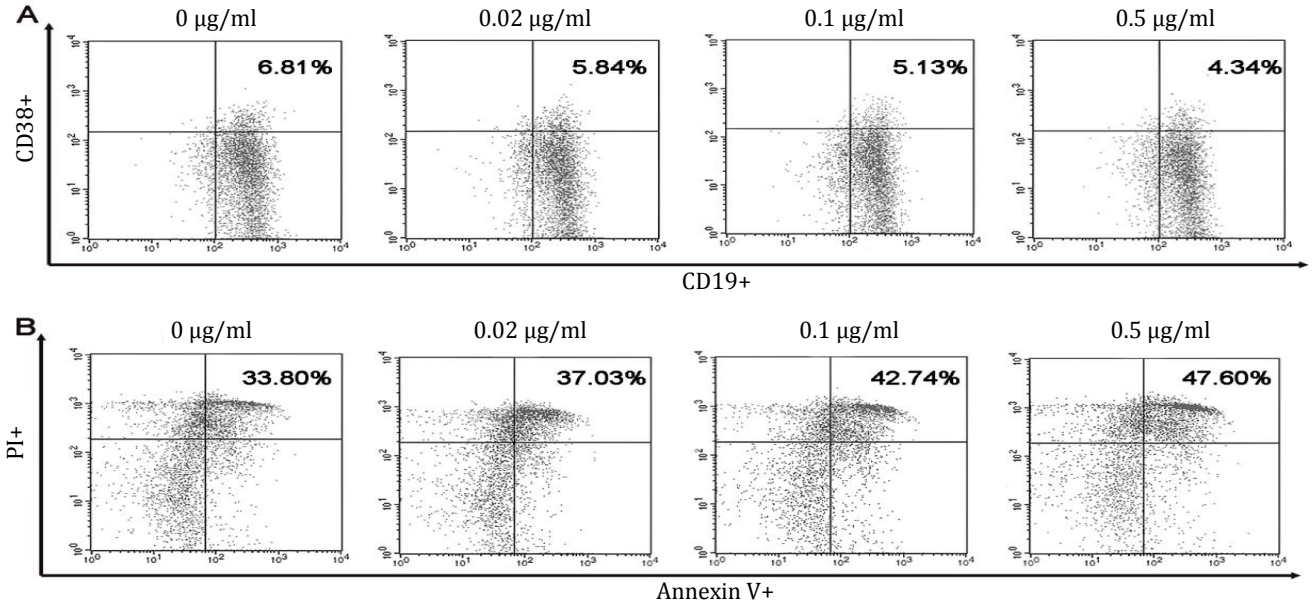

Figure 2. Dot plots of B cells maturation and apoptosis in vitro. Spleen of pristane-induced SLE BALB/c mice model cultured with BAFF, anti-CD40, LPS, IL-4 and without or with different doses of *B. pinnatum* leaves with ethanol extract (0, 0.02, 0.1, or 0.5 µg/ml). Cells were harvested and measured percentages of maturation (CD19+CD38+) (A) and apoptosis (Annexin V+PI+) (B) of B cells using flow cytometry

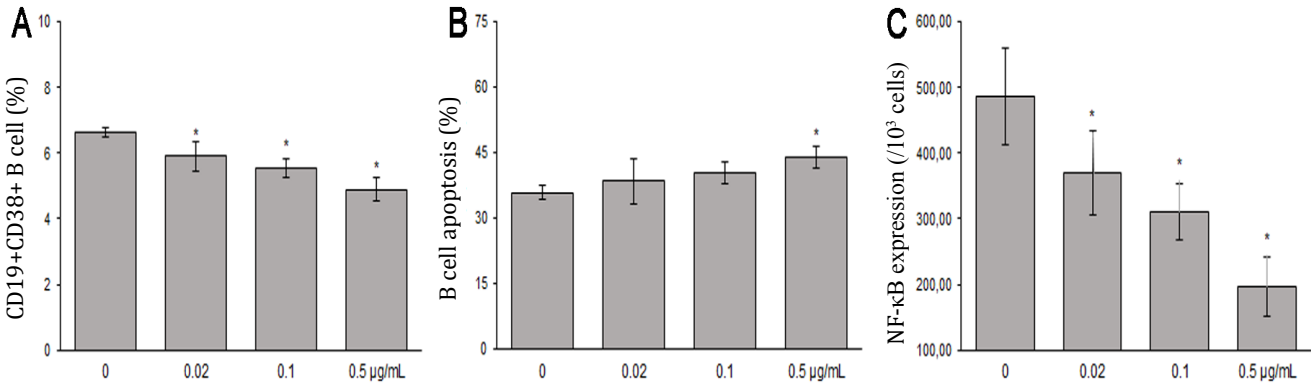

Figure 3. The effects of *B. pinnatum* leaves with ethanol extract on B cells *in vitro*. A) *B. pinnatum* leaves with ethanol extract inhibit maturation (CD19+CD38+) of B cells; B) *B. pinnatum* leaves extract promote apoptosis (Annexin V+/PI+) of B cells; C) *B. pinnatum* leaves with extract decrease expressions of NF-κB p65 (mean ± SD; n = 6, *p<0.05)

B. pinnatum leaves with ethanol extract decreased NF-κB p65 in pristane-induced SLE BALB/c mice B cells

B. pinnatum leaves with ethanol extract group had significant lower expressions of NF-κB p65 compared to the untreated group (p=0.013, p<0.01, and p<0.01, respectivelly). *B. pinnatum* leaves with ethanol extract decreased the expressions of NF-κB p65 (Figure 4).

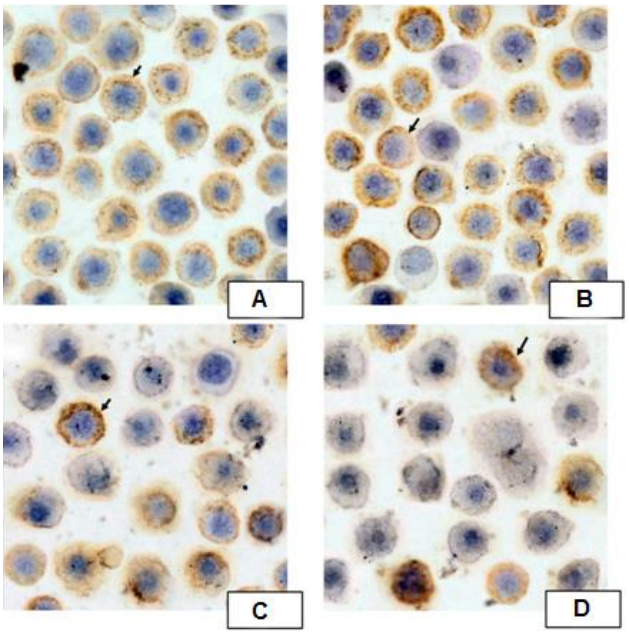

Figure 4. Expressions of NF-κB p65 by Immunocytochemistry. Spleen of pristane-induced SLE BALB/c mice model cultured with BAFF, anti-CD40, LPS, IL-4 and treated without or with different doses of B. pinnatum leaves with ethanol extract (0, 0.02, 0.1, or 0.5 μg/ml) for 72 hours. Cells were processed for immunocytochemistry using NF-κB p65 antibody as described in Materials and Methods

Correlations between the percentages of maturation, apoptosis, and expressions of NF-κB p65 of B cells

The percentages of maturation of B cells had moderate and significant correlation with apoptosis of B cells (r=-0.512, p<0.05). Significant and strong correlation was observed in the percentages of maturation and NF-κB p65 expressions of B cells (r=0.849, p<0.001). Significant and moderate correlation was observed in the percentage of apoptosis and NF-κB p65 expressions of B cells (n=6, r=-0.692, p<0.001) (Figure 5).

DISCUSSION

B. pinnatum is a wild plant that has been proven to have benefits as anti-infammatory, antitumor, and immunomodulatory agents.[11] Phytochemistry studies showed that *B. pinnatum* contains elements such as alkaloids, phenol, flavonoids, tannins, anthocyanin, glycosides, bufadienolide, saponins, coumarin, sitosterol, quinine, carotenoids, tocopherol, mucilago, lignin, and lectin.[13–15] The therapeutic effects of *B. pinnatum* in pristane induced SLE BALB/c mice B cells explored in this study. *B. pinnatum* leaves with ethanol extract decreased percentages of

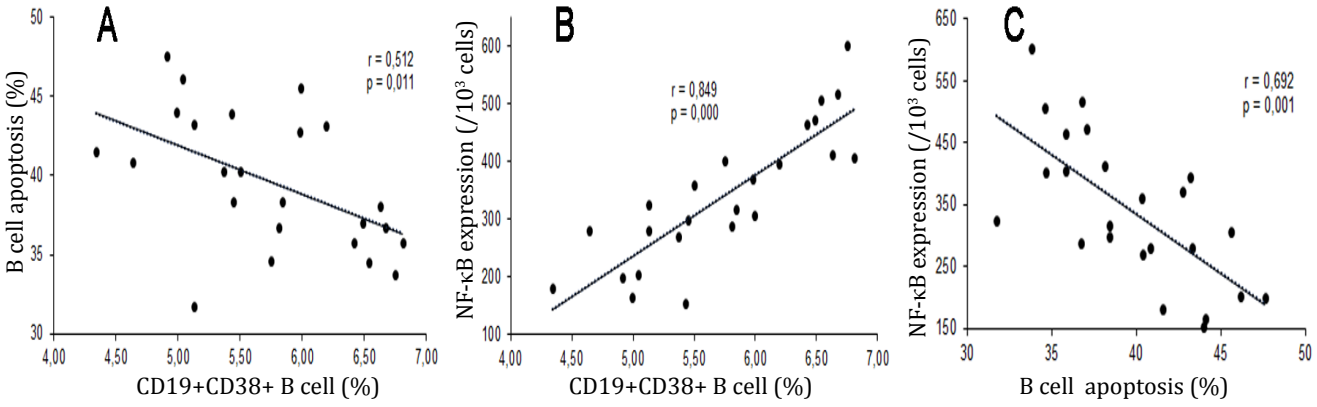

Figure 5. Correlations between percentages of maturation, apoptosis, and NF-κB p65 expressions of B cells. Diagrams showed correlations among the percentages of maturation (CD19+CD38+), apoptosis (Annexin V+PI+), and expressions of NF-κB p65 of B Cells. A) Significant and moderate correlation was observed in percentages of maturation and apoptosis of B cells (r=-0.512, p=0,011); B) Significant and strong correlation was observed in the percentages of maturation and expressionsNF-κB p65 of B cells (r=0.849, p=0,000); C) Significant and moderate correlation was observed in the percentage of apoptosis and expressionsNF-κB p65 of B cells (n=6, r=-0.692, p< 0,001)

maturation (CD19+CD38+) of B cells, increased the percentage of apoptosis (Annexin V+PI+), and decreased NF-κB p65 expressions in pristane-induced SLE BALB/c mice B cells *in vitro*.

The parenteral use of *B. pinnatum* has been granted permission by the German and Swissmedic E commission.[16] *B. pinnatum* preparations are available in tablets, powders, and parenterals (*Bryophyllum* 5%), produced by Weleda AG, and used as a tocolytic agent in premature pregnancy and various other medical conditions as well.[11] Previous study compared various parenteral agents and showed that administering of *Bryophyllum* 5% did not have significant effects to apoptosis and necrosis of lymphocytes and DC *in vitro.* However, this study showed different findings compared to Gründemann et al.[17] The administration of *B. pinnatum* leaves with ethanol extract increased percentages of B cells apoptosis.

Several strategies have been developed to suppress the production of autoantibodies by depletion of B cells, inhibition of B cells proliferation, and modulation of B cells. Recent studies were still focused on the mechanism of BAFF in depleting B cells.[18] This study performed B cells activation models by stimulating it with BAFF, IL-4, LPS, and anti-CD40 antibody. This study suggested that *B. pinnatum* leaves with ethanol extract could decrease B cells maturation, NF-κB expressions, and increase apoptosis of B cells. The limitation of this study was that it did not compare the effects of BAFF, IL-4, LPS, and anti-CD40 antibody stimulation respectively. Further research may require to understand the mechanisms of *B. pinnatum* metabolites in disrupting the ligand-receptor binding of B cells.

Bufadienolide is one of the main active metabolites in *B. pinnatum.* About 40.5–52 mg bufadienolide contained in every 100 grams of *B. pinnatum.*[12] Our unpublished in silico study was conducted to understand the affinity of bufadienolide active compounds (Bryophillin A, Bryophillin B, and Bryotoxin B) in *B. pinnatum* against BAFF and its receptors: BAFF-R, TACI, and BCMA. Docking results suggested that those *B. pinnatum* compounds interacted with BAFF-R, TACI, BCMA and BAFF through hydrogen bonds and hydrophobic interactions. These results showed that bufadienolide active compounds disrupted BAFF interactions with its receptors.

These study results demonstrated that single dose of pristane injection intraperitonialy in BALB/c mice induced arthritis, alopecia, and ascites after 16 weeks of injection. These results supported our previous study that manifestations of SLE such as arthritis, alopecia, and ascites appear in BALB/c mice model after single dose of 0.5 mg pristane injection intraperitonealy.[19] Based on the kinetics of autoantibody development following exposure to pristane, 16 weeks of the first clinical manifestation period was chosen.[20] The results showed that pristane induced immunity disregulation and induced autoreactive of B cells shown by producing autoantibodies like ANA.[19,21] ANA serum titersare increased in the week 16.

The clinical impact of this study is expected to find a new complementary therapy derived from the original leaves of Indonesia, *B. Pinnatum*, to improve the success of therapy in patients with SLE in Indonesia. However, this study still can not explain which specific secondary metabolic content has a role to the maturation and induction of B cell apoptosis. Besides, we also have not compared the effect of *B. pinnatum* leaves with ethanol extract between each stimulant to know specifically the role of secondary metabolite contained to the activation pathway of B cells development. The study would have yielded different results if a negative or normal control group had been included. Thus, further research should address this limitation in order to reveal more important findings.

In conclusion, *B. pinnatum* leaves with ethanol extract decreased the percentages of maturation (CD19+CD38+) of B cells, increased the percentage of apoptosis (Annexin V+PI+), and decreased NF-κB p65 expressions in pristane-induced SLE BALB/c mice B cells *in vitro*. Further studies in other lupus models and good-designed clinical trials are required to confirm these studies and identify its therapeutic effects, especially in humans.

Conflicts of interest

The authors affirm no conflict of interests in this study.

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Basic Medical Research

The production of SPusp45-MSP-1_{19} gene construct and its recombinant protein in *Lactococcus lactis* to be used as a malaria vaccine

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ABSTRAK

Latar belakang: Merozoite surface protein 1 *(MSP-1) adalah protein utama yang digunakan oleh Plasmodium selama invasi sel darah merah pada penderita malaria. MSP-1_{19}, salah satu jenis fragmen MSP-1, merupakan utas yang sangat lestari dan berpotensi sebagai kandidat vaksin malaria karena antibodinya mampu menahan invasi pada eritrosit secara in vitro. Penelitian ini bertujuan mengonstruksi gen dan mengekspresikan protein MSP-1_{19} pada Lactococcus lactis sebagai kandidat vaksin malaria.*

Metode: *Gen usp45-MSP-1_{19}, yang berasal dari hasil optimasi kodon dan gen sintetis, dimasukkan ke dalam vektor kloning pMAT. Vektor yang mengekspresikan fragmen MSP-1_{19} termasuk peptida sinyal usp45 (SPusp45) telah dikonstruksi dengan manipulasi DNA rekombinan menggunakan enzim restriksi. Protein MSP-1_{19} diekspresikan dengan presipitasi ammonium sulfat 45% dan dimurnikan dengan menggunakan kromatografi filtrasi gel Sephadex-G50. Ekspresi protein dianalisis dengan SDS-PAGE dan dot blot.*

Hasil: *SPusp45 dilengkapi gen MSP-1_{19} diamplifikasi dengan menggunakan primer tertentu dan berhasil dimasukkan ke dalam daerah* multiple cloning sites *(MCS) pada vektor ekspresi pNZ8148 dengan ukuran 3.538 pb sebagai vektor rekombinan. Protein MSP-1_{19} berhasil diekspresikan ke dalam Lactococcus lactis dengan berat molekul 10,45 kDa. Uji dot blot dari 3 jenis perlakuan berbeda menunjukkan hasil positif pada konsentrasi induksi nisin 10 ng/ml.*

Kesimpulan: *Penelitian ini menunjukkan gen usp45-MSP-1_{19} berhasil dimasukkan ke dalam daerah MCS vektor ekspresi pNZ8148 dan Protein MSP-1_{19} berhasil terekspresi dalam sistem NICE dari sel inang L. lactis.*

ABSTRACT

Background: Merozoite surface protein 1 (MSP-1) is a major protein used by the *Plasmodium* during red blood cells invasion in malaria. MSP-1_{19}, one of MSP-1 is highly conserved, and it is a potential malaria vaccine candidate because the monoclonal antibodies are capable blocking erythrocyte invasion *in vitro*. The aim of this study was to produce MSP-1_{19} gene construct and the recombinant protein in *Lactococcus lactis*.

Methods: Usp45-MSP-1_{19}, derived from codon optimization and the synthetic gene, was inserted into the pMAT cloning vector. A vector expressing MSP-1_{19} included usp45 has been constructed by the manipulation of recombinant DNA using restriction enzymes. The MSP-1_{19} protein was expressed to 45% ammonium sulfate precipitation and purified using Sephadex-G50 gel filtration chromatography. The expressed protein was characterized by SDS-PAGE and dot blot.

Results: usp45-MSP-1_{19} gene was amplified using specific primers and inserted into the multiple cloning sites in the expression vector pNZ8148 with size 3,538 bp as a recombinant vector. The protein of MSP-1_{19} was successfully expressed in *L. lactis* with molecular weight of 10.45 kDa. The dot blot was tested in 3 different comparisons between the host cells, non-induced cells, and induced cells with 10 ng/ml nisin. The results showed that 10 ng/ml nisin gave a positive reaction as detected by dot blot assay.

Conclusion: This study confirmed that the usp45-MSP-1_{19} gene was successfully inserted into the multiple cloning sites of the pNZ8148 expression vector and the MSP-1_{19} protein expressed in the NICE system of the *L. lactis* host cell.

Keywords: *Lactococcus lactis*, malaria, merozoite surface protein 1, nisin, usp45-MSP-1_{19}

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Malaria is one of the most contagious diseases that are still of a public health problem in the world including Indonesia. Malaria occurs in 106 countries. About 3.3 billion people live in endemic areas of malaria, and nearly 80% of malaria cases occur in the eastern part of Indonesia.[1] The prevalence of malaria reflected as annual malaria incidence (AMI) decreased in the period 2000–2004 from 31.09 to 21.2 per 1.000 population. In 2008, it fell to 18.82, and in 2010 it dropped to 12.27 per 1.000 population. Meanwhile, the prevalence of malaria annual parasite incidence (API) in 2000 was 0.81, and in 2004 fell to 0.15 per 1,000 population. In 2006, it increased to 0.19 then in the year 2007–2008 fell into 0.16 per 1,000 population.[2]

While currently artemisinin has been shown to be effective for malaria treatment, some degree of resistance has been reported to occur worldwide.[3] Therefore, a malaria vaccine is likely to be used as a potential tool for malaria control. Merozoite surface protein 1 (MSP-1) is a potential candidate of malaria vaccine because the monoclonal antibodies raised against this protein show its capability to block erythrocyte invasion *in vitro*.[4] The MSP-1 is a major protein used by the *Plasmodium* during red blood cells invasion.[5] Merozoite is the smallest cell measuring 1–2 μm in the life cycle of *Plasmodium*.[6] The MSP-1 contains 2 epidermal growth factors (EGF-like domain) which are important parts during erythrocyte invasion.[7,8] MSP-1_{19} gene sequence located on block 17 is highly conserved. Therefore, it is used for the development of malaria vaccine.[9]

Lactococcus lactis has a generally recognized as safe (GRAS) host cell status and is often used in expressing recombinant proteins. *L. lactis* has been characterized as a good microorganism for the industry and capable of secreting recombinant protein into its growth media, so that the resulting product is free of endotoxin. The construction and expression of the MSP-1_{19} gene in *L. lactis* has been prepared before.[7] In the study, MSP-1_{19} native gene construct was made by the fusion of SlpA peptide signals and intracellular protein expression. However, the level of MSP-1_{19} protein expression is low. Estimated protein MSP-1_{19} produced are only a few ng per 10^7 bacterial cells.

In addition, protein isolation is done through cell lysis. Since proteins are still at intracellular level, protein extraction process becomes more difficult. The fusion of the SlpA peptide signal should be able to bring the MSP-1_{19} protein out through the cell membrane, so it can be secreted to the extracellular. In this study, the construction and expression of MSP-1_{19} gene that has undergone codon optimization fused with usp45 signal peptide has been transformed into *L. lactis*. The usp45 signal peptide allows the MSP-1_{19} protein to be secreted into the extracellular compartment. The usp45 signal peptide is an N-terminal signal peptide capable of optimizing the translocation of precursor proteins from within cells to the outside of the *L. lactis* cell wall.[10] The codon optimization is performed to remove the biased codons that can inhibit the production of recombinant proteins in the prokaryotic system. It is a strategy to improve the production efficiency of recombinant proteins in the prokaryotic system. The purpose of this study was to construct the expression vectors for the MSP-1_{19} gene and to express MSP-1_{19} recombinant protein *L. lactis* as a candidate for malaria vaccine.[10]

METHODS

Genes, plasmids, bacteria, growth media, and primers used in this study

The material used in this study is the synthetic gene usp45-MSP-1_{19} (GeneBank: AF165928) which is the result of codon optimization and has been ligated into the plasmid pMA-T. The growth media are *Luria-Bertani (LB)* and M17B bacteria. Plasmid used is pMA-T (cloning vector), and pNZ8148 is used as expression vector. Several vector and bacterial strains can be seen in Table 1, as well as several the primer pairs used in this study: forward ProNisA primer with TTCCCTCGAGGGATCTATGTC sequence (restriction site *Nco1*) and terminator reverse pNZ8148 primer with TGCTTTATCAACTGCTGC sequence (restriction site *Xba1*). *E. coli* was grown in *Luria-Bertani* (LB) medium (10 g/l tryptone [Oxoid, England], 10 g/l sodium chloride [Merck, Denmark], 5 g/l yeast extract [Oxoid, England]) supplemented with 100 μg/ml ampicillin (Bio Basic, Canada) at 37°C with agitation. Monoclonal antibody used for dot blot hybridization assay was *Plasmodium falciparum* MSP1 antibody (PEM-1) from Thermo Fisher. MSP-1 is highly conserved among broad *Plasmodium* species. Thus, this antibody could also detect MSP-1 from *P. yoelii.*

Construction of expression vector

The construction of the expression vector began by isolating the pMA-T plasmid. The MSP-1_{19} gene was obtained by digesting the fragment of interest using restriction enzymes on the *Nco1* and *Xba*1 sites, followed by gel purification. Pure DNA fragments of MSP-1_{19} were ligated into pNZ8148 expression plasmid using T4 DNA ligase enzyme prior to introduction into *E. coli* MC1061. The transformation to *E. coli* MC1061 was performed by the heat shock method.[11]

Furthermore, pNZ8148-usp45-MSP-1_{19} plasmid in *E. coli* MC1061 was isolated and subsequently transformed into *L. lactis*. Competent cells were grown in 0.2% *L*. *lactis* in 5 ml M17 broth + 0.5% glucose overnight at 30℃. Subsequently, the culture was incubated in 25 ml M17 broth containing 0.5% Glucose, 0.5 M sucrose, and 2.5% glycin overnight at 30℃. The culture was then put into 75 ml of M17 broth containing 0.5% glucose, 2.5% glycine, and 0.5 M sucrose and grown until the culture reached OD600 ~ 0.2–0.3 prior to harvesting the cell using centrifuge. Cells were resuspended with 0.5 M sucrose +10% glycerol. The transformation process was carried out using Gene Pulser BIORAD electroporation at 2,000 V, 25 µF, 260 Ω. The success of the transformation process of the pNZ8148-usp45-MSP-1_{19} construct into *L. lactis* was confirmed by amplification of the bacteria colony by polymerase chain reaction (PCR), plasmid isolation followed by restriction enzyme digestion, and sequencing of inserts or plasmid construct.

pNZ8148-usp45-MSP-1_{19} plasmid in *L. lactis* was grown further to increase the expression of the recombinant protein.[12] *L. lactis* carrying recombinant plasmid was grown in 5 ml of M17 medium that contained 0.5% glucose. A total of 1.5 ml of *L. lactis* culture was subjected by centrifugation at 10.000x g for 2 min. The pellet was resuspended with solution I containing 10 mM EDTA (pH 8.0), 25 mM Tris/HCl, 50 mM glucose, and 20 mg/ml lysozyme (Sigma) was added and subsequently incubated for 15 min at 37°C. The mixture was added with 300 µl fresh solution II containing 0.2 M NaOH, 2% SDS and incubated for 3 min at room temperature. As much as 170 µl solution III containing 1.2 M Tris/HCl (pH 7.0) and 2 M NaCl were added to the mixture. The mixture was subsequently homogenized with 500 µl phenol. The upper layer phase was mixed with 600 µl PCI (Phenol Chloroform Isoamyl alcohol) then it was centrifuged. Moreover, 600 µl of isopropanol was added and then subjected to incubation on ice for 15 min. The mixtures were subjected to centrifugation and washed with 70% ethanol. The pellet was dried and dissolved in 20 µl ddH2O and 5 µl of 1 mg/ml RNase. The results of plasmid isolation were then subjected to *Nco1* and *Xba1* restriction enzymes, PCR of the colonies, and DNA sequencing to confirm the inserts sites. The schematic construction representation of the cloning vector pMAT and expression vector pNZ8148 which was expressed as a fusion protein pNZ8148- usp45-MSP-1_{19} can be seen in Figure 1.

Expression and purification of recombinant protein MSP-1_{19}

Expression of MSP-1_{19} as heterologous protein inside *L. lactis* was conducted with nisin inducible controlled expression (NICE) system. Protein expression was performed by the following methods. The MSP-1_{19} expression in *L. lactis* was started by inoculating 10% fresh culture into 100 ml M17 broth containing 10 µg/ml chloramphenicol and 0.5% glucose without agitation at 30℃ for 10 hours to reach OD600 ~ 0.5. Furthermore, induction was performed using 10 ng/ml nisin and incubated for 5 hours at 30°C. The cells were subjected to centrifugation

Table 1. Bacterial strains and vector used in this study

Strains and vector	Relevant characteristics	Source of reference
Bacterial strains		
Escherichia coli MC1061	Bacterial/host strain for cloning	MoBiTec
Lactococcus lactis NZ3900	Bacterial/host strain for expression	MoBiTec
Vector		
pMA-T	Cloning vector containing ampicillin resistance gene	Invitrogen
pNZ8148	The nisin-induced expression vector containing chloramphenicol resistance gene	MoBiTec

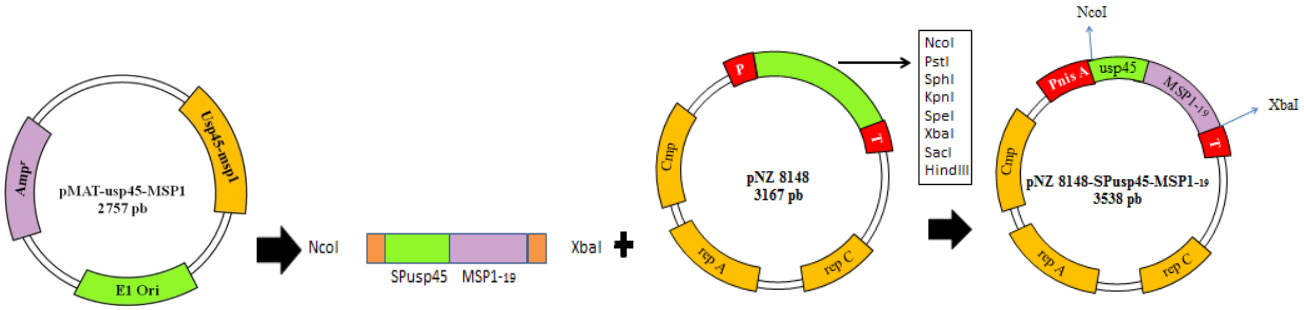

Figure 1. Schematic representation of the pNZ8148-usp45-MSP-1_{19} expression construct containing the pMAT cloning vector and pNZ8148 expression vector

at 10.000 rpm at 4°C for 30 min. The recombinant protein was subjected to precipitation with 45% ammonium sulfate 27.7 g and subsequently incubated at 4°C for 10 hours. The cells were subjected to centrifugation to form the pellet for further elution step with 50 mM Tris HCl pH 7.4. The pellet suspension was purified by stationary phase adsorption chromatography Sephadex-G50. A total of 1 mL of pellet suspension was incorporated into chromatographic column, and then eluted with 50 mM Tris HCl pH 7.4.[13,14]

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The expressed proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE analysis using 10% separation gel and 4% retaining gel to examine the molecular weight of MSP-1_{19}. The recombinant protein was mixed with loading dye buffer (containing 50 mM Tris HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoetanol, 12.5 M EDTA, 0.02% bromophenol blue in distilled water) and was subjected to heating at 95°C for 10 min prior to SDS-PAGE gel electrophoresis at 100 V and 20 mA. The gel was stained with silver staining kit (Fermentas Silver Stain Kit).

Determination of total protein concentration

The total protein concentration was quantified with bicinchoninic acid (BCA) kit using standard bovine serum albumine (BSA) curve. The standard concentrations of BSA used were 25 µg/ml, 125 µg/ml, 250 µg/ml, 500 µg/ml, 750 µg/ml, 1,000 µg/ml, 1,500 µg/ml, and 2,000 µg/ml. Working reaction was made by mixing reagents A and B with a ratio of 50:1. The sample and working reaction were added in a microplate 96-well by sample:working reaction ratio of 1:20. Microplate was incubated at 37°C for 30 minutes, and then the reaction result was read at 540 nm wavelength using enzyme-linked immunosorbent assay (ELISA) reader (Thermo Scientific Multiskan EX Microplate Reader).

Dot blot hybridization

Ten µl of protein samples were spotted on top surface of the membrane prior to blocking with 10% skim milk in 1x tris-buffered saline (TBS) for 1 hour. The membrane was washed 3 times, for 15 min, 5 min, and 5 min with 0.1% TBS-Tween. Furthermore, the membrane was incubated with monoclonal MSP1 antibody for 1 hour, and washed again 3 times with 0.1% TBS-Tween. The IgG mouse alkaline phosphatase conjugates as a secondary antibody was used to incubate the membrane for 1 hour prior followed by the washing step. Staining was performed using nitro blue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate NBT-BCIP substrate solution. The membrane was immersed in BCIP/NBT solution (500 µl NBT solution, 500 µl BCIP solution, dan 4 ml distilled water) for 1-5 min at room temperature until the color was developed. When the dot blots were detected, the incubation process was stopped by soaking the membrane in distilled water.[15,16]

RESULTS

The gene used in this study was the synthetic gene usp45-MSP-1_{19} that has been ligated into the pMAT plasmid. The first stage of making the expression construct was the isolation of pMAT-usp45-MSP-1_{19} plasmid with *Nco*1 and *Xba*1 restriction enzymes to release the gene/insert from the plasmid. The usp45-MSP-1_{19} gene was

then separated from the plasmid using restriction analysis as shown on agarose gel (Figure 2).

Figure 3 describes the next stage was the ligation of the usp45-MSP-1_{19} gene into the pNZ8148 expression vector. The 371 bp usp45-MSP-1_{19} gene was successfully inserted into the multiple cloning site (MCS) of the pNZ8148 expression vector, forming a 3,538 bp recombinant plasmid. The recombinant vector was confirmed by *Nco*1 and *Xba*1 restriction enzymes. Ligation of the usp45-MSP-1_{19} gene and the pNZ8148 vector was successfully transformed into *E. coli* MC1061.

The pNZ8148- usp45-MSP-1_{19} recombinant vector was validated by DNA sequencing to determine whether or not mutations of the inserted MSP-1_{19} gene were present. The recombinant plasmid containing pNZ8148-usp45-MSP-1_{19} was 3538 bp in size, encoding 124 amino acids, with a starting point of transcription from 47 nucleotides prior to the usp45-MSP-1_{19} gene and ended at 559 nucleotides after the usp45-MSP-1_{19} gene (Figure 4). The usp45-MSP-1_{19} gene was cloned on the *Nco*1 site, causing the addition of 2 amino acids (6 nucleotides) during translation.

In this study showed the different concentration of total protein. The highest concentration of total protein was 0.936±0.04 mg/ml for cells that were induced with 10 ng/ml nisin. However, this was not significantly different with the concentration of total non-induced protein (0.549±0.03 mg/ml). The lowest concentration was the vector alone (0.188±0.04 mg/ml).

MSP-1_{19} molecular weight was 10.45 kDa (Figure 5A) suggesting that the protein was expressed and secreted out of the cell due to addition of the usp45 signal peptide that was able to recognize the signal recognition particle (SRP). The expression of MSP-1_{19} protein was shown by dot blot analysis tested by the MSP-1_{19} monoclonal antibody as shown by the purple spot in the membrane (Figure 5B).

DISCUSSION

The expression vector construction in this study was initiated by the ligation of the usp45-MSP-1_{19} synthetic gene into the pMAT plasmid. The inserts were verified using *Nco*1 and *Xba*1

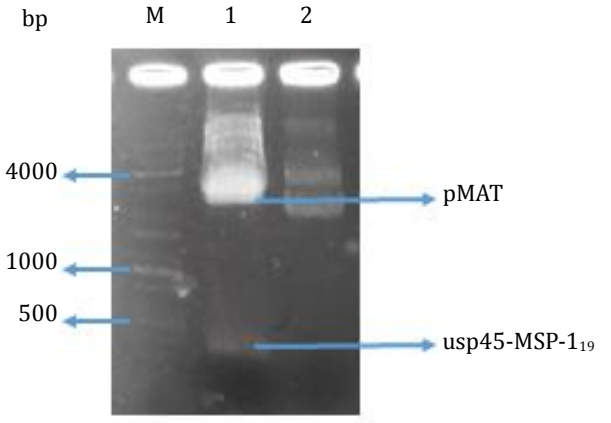

Figure 2. Restriction analysis of pMAT-usp45-MSP-1_{19} recombinant plasmid M: 1kb DNA ladder marker, lane 1: digested pMAT plasmid of 2,757 bp, lane 2: undigested usp45-MSP-1_{19} of 371 bp

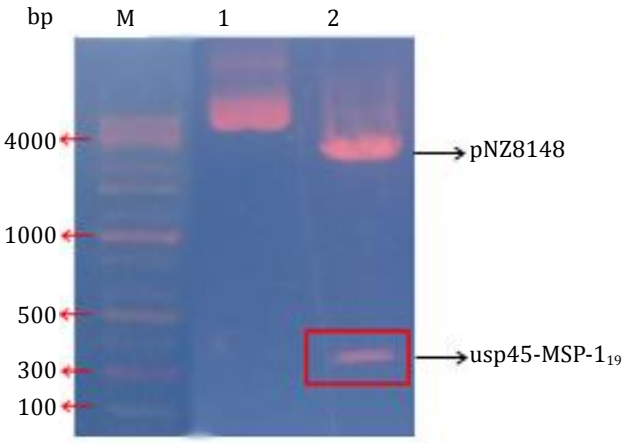

Figure 3. Confirmation of pNZ8148-usp45-MSP-1_{19} recombinant protein by restriction enzyme cutting. Lane 1: undigested recombinant plasmid of pNZ8148 containing usp45-MSP-1_{19} gene. Lane 2: pNZ8148- usp45-MSP-1_{19} recombinant protein cut with *Nco1* and *Xba1* enzymes

restriction enzymes to separate plasmids and genes. The ligation of the usp45-MSP-1_{19} gene into the pNZ8148 expression vector was done. The 371 bp usp45-MSP-1_{19} gene was successfully inserted into the multiple cloning sites (MCS) in the pNZ8148 expression vector. Thus, it formed a recombinant protein of 3,538 bp.

The recombinant vector pNZ8148-usp45-MSP-1_{19} was introduced into *L. lactis* NZ3900 using electroporation technique. This technique was used to insert the foreign DNA into a host of gram-positive bacteria that had the characteristic

```
   1  CCCCATTTTGGAATAGAACATTAACAAATCTAAAACAGTCTTAATTCTATCTTGAGAAAGTATTGG   66

                                                        -35
  67  TAATAATATTATTGTCGATAACGCGAGCATAATAAACGGCTCTGATTAAATTCTGAAGTTTGTTAG  132

        -10         +1                                RBS          Nco1
 133  ATACAATGATTTCGTTCGAAGGAACTACAAAATAAATTATAAGGAGGCACTCACCATGGCCATGAA  198
   1                                                              M  A  M  K    4

 199  AAAAAAGATTATCTCAGCTATTTTAATGTCTACAGTGATCTTAAGTGCTGCAGCCCCGTTGTCAGG  264
   5   K  K  I  I  S  A  I  L  M  S  T  V  I  L  S  A  A  A  P  L  S  G   26

 265  TGTTTACGCCGGTGTTGATCCAAAACATGTTTGTGTTGATACACGTGATATTCCAAAAAATGCTGG  330
  27   V  Y  A  G  V  D  P  K  H  V  C  V  D  T  R  D  I  P  K  N  A  G   48

 331  TTGTTTTCGTTATGATAATGGTAATGAAGAATGGCGTTGTTTATTAGGTTTCAAAAAAGAAAACAA  396
  49   C  F  R  Y  D  N  G  N  E  E  W  R  C  L  L  G  F  K  K  E  N  N   70

 397  TACATGTGTTGAAGATAATAATCCAACATGTGATACAAATAATGGTGGTTGTGATACAGCTGCTTC  462
  71   T  C  V  E  D  N  N  P  T  C  D  T  N  N  G  G  C  D  T  A  A  S   92

 463  ATGTCAAACAGGTGATCGTTCAGGTGAAAATTCAAAAAAAGTTATTTGTACATGTAAAGAACCAAC  528
  93   C  Q  T  G  D  R  S  G  E  N  S  K  K  V  I  C  T  C  K  E  P  T  114

 529  ACCAAATGCTTATTATGAAGGTGTTTTTTGTTAATCTAGAGAGCTCAAGCTTTCTTTGAACCAAAA  594
 115   P  N  A  Y  Y  E  G  V  F  C  *** Xba1                           124

 595  TTAGAAAACCAAGGCTTGAAACGTTCAATTGAAATGGCAATTAAACAAATTACAGCACGTGTTGCT  660

 661  TTGATTGATAGCCAAAAAGCAGCAGTTGATAAAGCAATTACTGATATTGCTGAAAAATTGTAATTT  726

                      Terminator
 727  ATAAATAAAAATCACCTTTTAGAGGTGGTTTTTTTATTTATAAATTA                     773
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Figure 4. Sequencing analysis pNZ8148- usp45-MSP-1_{19} protein recombinant. NisA -35, -10, 1: are the starting points of transcription, RBS is the ribosome binding region (Shine-Dalgarno) and terminator is the end point of transcription. The green color is the restriction site (*Nco1/Xba1*), the blue color is Usp45 signal peptide, the red color is merozoite surface protein 1 (MSP-1_{19}), and the three asterisk is the stop codon

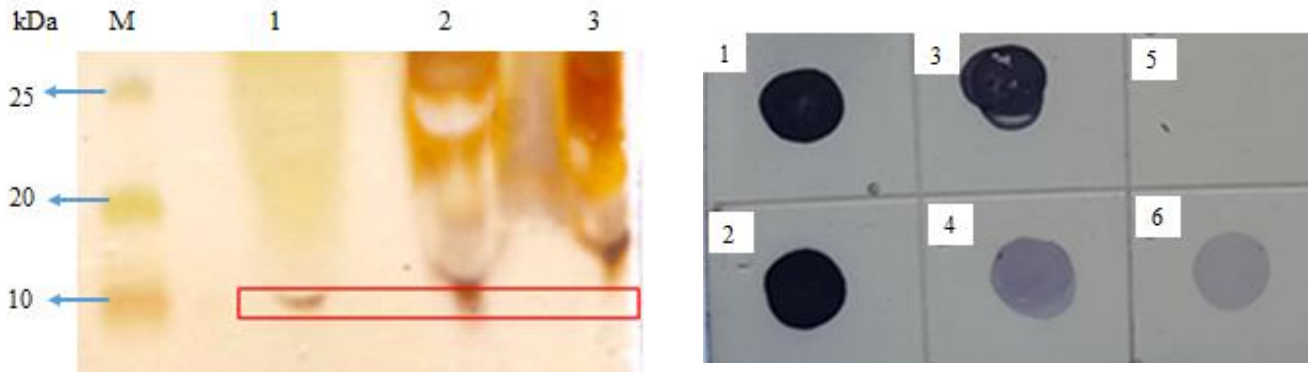

Figure 5. Expression and purification of the MSP-1_{19} fusion protein. A) SDS-PAGE of target protein expression and purification under different comparisons of induction, non-induced, and the lysed host cell. Molecular weight marker has shown in the left lane; lane 1: induced with 10 ng/ml nisin; lane 2: non-induced; lane 3: *Lactococcus lactis*; B) Dot blot analysis. Boxes 1, 2 and 3 are MSP-1_{19} dot blots induced with 10 ng/ml nisin; Boxes 4 and 6 are MSP-1_{19} dot blots non-induced; Box 5 is the dot blot of *Lactococcus lactis*

of a thick cell membrane structure.[17] This electroporation technique applied high electrical voltage passed in a short time. Thus, it formed a pore on the cell membrane that could be inserted with foreign DNA. Selection of transformants using chlorampenicol antibiotics was possible because the vector harbors, the chloramphenicol-resistant genes, was used as selection marker. The addition of sucrose and glycine can help the transformation process to become more efficient.[18]

The ligation of usp45-MSP-1$_{19}$ gene into pNZ8148 vector which was successfully introduced into *E. coli* host MC1061 was drawn from research by Wu et al.[12] They introduced the vector into *E. coli* first and then to the *L. Lactis,* so that the efficiency of the transformation to *L. lactis* was high. The pNZ8148 expression vectors are known to not have a high vector replication level, but it is balanced by their two starting points of replication, repA and repC, which are easily recognized by *E. coli* and *L. lactis*, respectively.[19]

Referring to Wu et al,[12] the gene inserted into the expression vector was first adapted to the addition of two nucleotide bases of CC after translational starting site fused with *Nco1* restriction sites (CCATGG). This is because the *Nco*1 site has already contained the ATG as the starting point of translation leading to mistranslation start site at the *Nco*1 site. At the usp45-MSP-1$_{19}$ signal peptide gene sequence in the pNZ8148 vector (Figure 4), there was NisA -35, -10, +1 as the transcription beginning of the ribosome-binding region/RBS (Shine-Dalgarno), TAA as the stop codon and it was terminated by a terminator as the ending point of transcription.[20]

The usp45-MSP-1$_{19}$ gene was expressed in the nisin inducible expression (NICE) system. The expression of the MSP-1$_{19}$ recombinant protein was performed by the induction of 10 ng/mL nisin. Giving nisin as an inducer from the outside the cells will stimulate the start of transcription at the nisA promoter through nisK and nisR regulon integrated in chromosomal DNA of *L. lactis*.[21] Possible expression level is controlled by the concentration of nisin given from the outside. The results of this response will form a transcription factor that activates the promoter nisA, so that the transcription process by RNA polymerase can be functioning.[22]

The presence of target protein bands in non-induced samples is expected due to basal activity causing the usp45-MSP-1$_{19}$ gene to be expressed even in the absence of nisin induction. This has been demonstrated in the Mohseni et al[21] study that NICE expression systems often exhibit basal expression activity (in the absence of induction) when expressed in *Lactobacillus*. In addition to adjusting the dose of nisin, several other ways can be used to increase the gene expression level, such as by substituting an inducible promoter to a constitutive promoter. In accordance to the results of Lages et al,[23] the administration of 10 ng/ml nisin induction in *L. lactis* increased gene expression, resulting in more protein than the non-induced. The concentrations of nisin has compared in the range between 0.5 and 5 ng/ml to induce the expression genes of antibacterial in *L. lactis*, and obtained the result that the highest of antibacterial was produced by *L. lactis* induced with nisin at 5 ng/ml by Mustopa et al.[24] Moreover, codon optimization can also be performed. Nisin concentration required for induction is very low, between 0.5–10 ng/ml, while the high concentration of nisin of more than 10 ng/ml used will have negative effect on the host because it can suppress the growth of *L. lactis*.[25]

As stated in the results section, usp45 signal peptide was able to recognize signal recognition particle (SRP) indicated by the protein being successfully secreted outside the cell to the growing medium. The SRP will bring the pre-peptide to the SRP receptor present in the membrane, which then will be brought to the protein translocation channel (PTC). The signal peptide opened the PTC, then the mature peptide was removed whereas the usp45 signal peptide would be cut by the peptidase.

Detection of MSP-1$_{19}$ protein was also seen from the dot blot hybridization test which is an immunological test whose function is similar to the western blot to detect the specificity of the reaction between the antigen and the antibody. In the samples induced with 10 ng/ml nisin, the positive results of the dot blot test were observed (Figure 5B). The results showed specific reactions between MSP-1$_{19}$ proteins with MSP1 monoclonal antibodies, seen from color developed on the membrane. Characterization of immunogenicity of primary antibodies tested by dot blot assays

showed the result of 10.45 kDa MSP-1_{19} antigen was reacted with anti-MSP1 specific antibodies.

In conclusion, this study showed that MSP-1_{19} protein fused with usp45 as signal peptide has been successfully constructed and expressed inside the GRAS organism in *L. lactis.* This heterologous expression of MSP-1_{19} protein by GRAS host cell can be expected as the candidate for mucosal malaria vaccine which could induce both mucosal and systemic responses. Although initial test of expressed MSP-1_{19} with monoclonal antibody *in vitro* has showed a positive result, *in vivo* investigation related to the efficacy of induced antibody should be determined.

Conflicts of Interest

The authors affirm no conflict of interest in this study

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Clinical Research

Association between varicocele grade and semen analysis parameter

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ABSTRAK

Latar belakang: *Varikokel merupakan suatu kondisi yang dicirikan oleh pemanjangan dan dilatasi pleksus pampiniform. Sekitar 50% kasus infertilitas laki-laki disebabkan oleh varikokel. Varikokel dapat mempengaruhi komponen sperma. Penelitian ini bertujuan untuk menentukan hubungan antara derajat varikokel berdasarkan pemeriksaan ultrasonografi (USG) Doppler dengan konsentrasi sperma, motilitas sperma dan morfologi sperma berdasarkan pemeriksaan analisis sperma.*

Metode: *Penelitian dilakukan dengan metode deskriptif potong lintang. Pengambilan data berupa data sekunder yaitu 85 penderita varikokel yang datang berobat ke Departemen Urologi RS Ciptomangkusumo, kemudian melakukan pemeriksaan ultrasonografi dilakukan di Departemen Radiologi RS Cipto Mangunkusumo, Jakarta dan pemeriksaan analisis sperma di Departemen Obstetri dan Ginekologi RS Cipto Mangunkusumo, Jakarta.*

Hasil: *Terdapat hubungan antara derajat varikokel kanan maupun kiri dengan morfologi sperma, konsentrasi sperma dan motilitas sperma (p<0,05). Hubungan bermakna juga ditemukan antara kondisi maksimal dengan komponen analisis sperma tersebut.*

Kesimpulan: *Derajat varikokel mempengaruhi komponen analisis semen.*

ABSTRACT

Background: Varicocele is a condition characterized by elongation, dilatation and tortuousity of spermatic vein in pampiniform plexus. Approximately 50% of infertility cases among men are caused by varicocele. The varicocele may affect the components of sperm. This study aimed to determine the association between varicocele grade based on ultrasound Doppler examination and sperm concentration, sperm motility, and sperm morphology based on semen analysis examination.

Methods: This was a descriptive, cross-sectional study which used secondary data from 85 patients that visited Department of Urology, Cipto Mangunkusumo Hospital, then underwent ultrasonography examination at Department of Radiology, Cipto Mangunkusumo Hospital and semen analysis examination at Department of Obstetrics and Gynecology, Cipto Mangunkusumo General Hospital.

Results: Varicocele grade was significantly associated with sperm morphology, concentration and motility (all p<0.05). Significant association was found between maximum condition and semen analysis component.

Conclusion: Varicocele grade may affect semen analysis component.

Keywords: semen analysis, ultrasound examination, varicocele's grade

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Varicocele is a condition characterized by elongation, dilatation and tortuosity of spermatic vein in pampiniform plexus. The incidence of varicoceles in the general population varies from 4.4–22.6%, whereas in men with primary and secondary infertility, the incidence is 21–41% and 75–81%, respectively.[1] It is the most frequent abnormal physical finding among men who undergo infertility evaluation.[2] Varicocele can influence all of semen parameters such as sperm concentration, sperm motility and sperm morphology.[1–3]

Al Ali et al found decreased quality of semen as well as higher level of testosterone in patient with varicocele grade III. Similarly, Mori et al also reported varicocele grade II and grade III caused decrease in sperm quality and testes volume. However, there were no significant differences between both grades. The study also mentioned that Grade III varicocele adolescents were much closer to the World Health Organization (WHO) sperm motility cut off value of which is established in 1999. Despite of that, sperm quality deterioration in varicocele is progressive and expected to cross into the WHO-defined infertile range much sooner.[2,4]

Approximately 83% of varicocele patients were under 50 years of age, and about 75% of patients reported infertility as the chief complaint and uncomfortable sensation at scrotum as additional symptom.[5] The study also found that all of the patients had varicocele in the left side and 62.5% of patients presented oligozoospermia. There was no linear association between varicocele grades and sperm concentration, whereas testes atrophy had direct correlation with varicocele grades. In additional, 38.5% of varicocele patients had one child or more, but 61.5% of patients had no child.[2,4,5]

Up to nowadays, studies regarding association between varicocele grade and disturbance of semen analysis component are still scarce, especially in Indonesia. Referring to the latest WHO sperm analysis guideline established in 2010, it is confirmed that sperm analysis cut off has been updated. Therefore, this study may provide new paradigm and different analysis. This study aimed to determine the association of varicocele grade with sperm concentration, motility and morphology.

METHODS

This was a cross-sectional study conducted at Cipto Mangunkusumo Hospital between May 2013 to February 2014 with ethical approval number 584/H2.F1/ETIK/2013. Medical records of patients aged above 16 years old who were clinically diagnosed as varicocele during October 2013 to January 2014 were evaluated. The inclusion criteria included patients who were diagnosed with varicocele by physical examination who had undergone testis ultrasonography examination as well as semen analysis. Patient who had undergone varicocelectomy, abnormal follicle-stimulation hormone (FSH) level (FSH serum level out of 1–14 IU/L), small testes volume (testes volume below 13.9–18.9 ml^3 with difference between left and right testes more than 20% according to ultrasonography examination), and the long interval duration between ultrasonography (USG) and semen analysis (more than 1 month) were excluded.

In this study, varicocele was graded using US-based criteria as follows: grade 0, if the diameter of pampiniform plexus is less than 2 mm during Valsalva and non Valsalva maneuver; grade I, if the diameter of pampiniform plexus is less tahn 2 mm during non-valsalva, but 2–3 mm during valsalva maneuver; grade II, if the diameter of pompiniform plexus is 2–3 mm during both condition; and grade III, if the diameter is more than 3 mm during the Valsalva and non-valsalva meneuver.[6]

The analysis of sperm quality using three components: sperm concentration, motility, and morphology. Sperm concentration is reported as azoospermia, oligozoospermia, and normozoospermia. Sperm motility is sorted as azoospermia, asthenospermia, and normozoospermia. Sperm morphology is sorted as azoospermia, teratozoospermia, and normozoospermia. According to WHO laboratory manual for sperm examination (2010), the terms mentioned before are defined based on criteria as follow: azoospermia is a condition of absence of sperm in the semen. Oligozoospermia is a condition of sperm concentration less than 15 million per ml semen. Asthenozoospermia is a condition of

progressively motile sperm less than 50% of total sperm. Teratozoospermia is a condition of sperm with normal morphology less than 30% of total sperm. Normozoospermia is a condition of sperm with concentration above 39 million per ml semen, progressively motile sperm is more than 50% of total sperm, and morphologically normal sperm is above 30% of total sperm.[7]

Varicocele grade data measurement was analyzed according to diameter of the right testis group and left testis group. But, varicocele grade may vary between left and right testis. This condition may affect result of overall analysis. So, it is necessary to add maximum condition group which is drawn from either left or right pampiniform plexus with highest number in diameter in each individual.

The numerical data were presented as means ± standard deviation. McNemar's test was used for comparison of the scored quality of the examination. All statistical analyses were performed using statistical product and service solutions (SPSS) 11.5 for Windows.

RESULTS

A total of 85 patients were recruited in this study. The mean age of the subjects was 35±6.27 years old. The subjects mostly came from Jakarta-Bogor-Depok-Tangerang-Bekasi (83.5%). Maximum

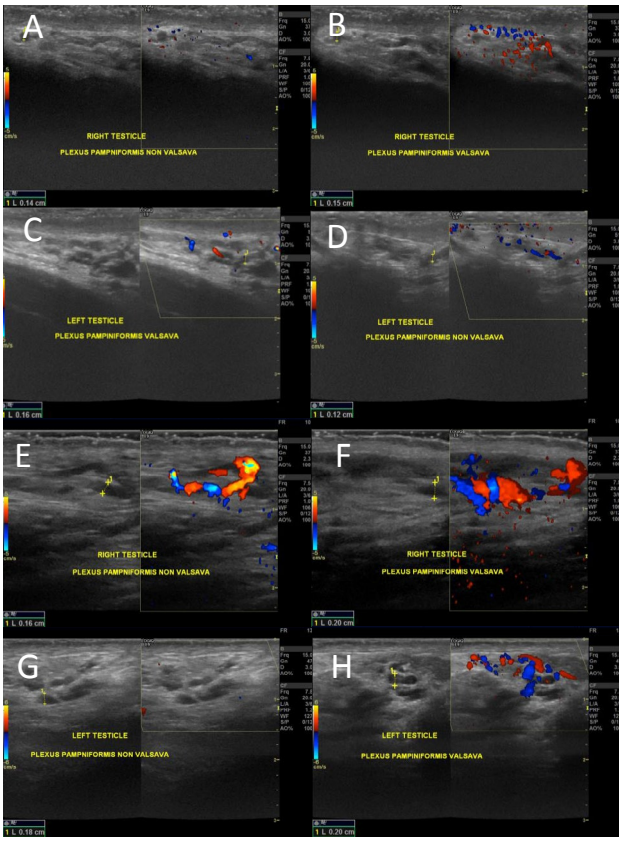

Figure 1. Doppler ultrasound of grade 0 varicocele. A is the right testicle and C is the left testicle. Both showing plexus pampiniform diameter below 2 mm in relaxed supine position. B and D showing both left and right pampiniform plexus increased in diameter below 2 mm. Doppler ultrasound of grade I varicocele. E is the right testicle and G is the left testicle. Both showing plexus pampiniform diameter below 2 mm in relaxed supine position. Both right (F) and left (H) pampiniform plexus diameter increased between 2–3 mm during Valsava maneuver

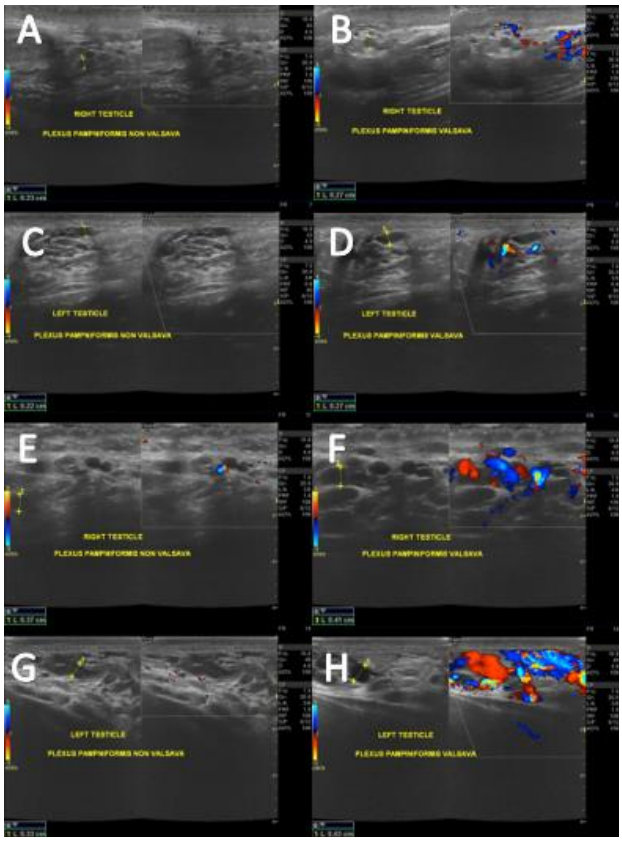

Figure 2. Doppler ultrasound of grade II varicocele. A) is the right testicle and C) is the left testicle. Both showing plexus pampiniform diameter at less than 3 mm in relaxed supine position. Both right (B) and left (D) pampiniform plexus diameter increased at the range of 2-3 mm during Valsava maneuver. Doppler ultrasound of grade III varicocele. E) is the right testicle and G) is the left testicle. Both showing plexus pampiniform diameter more than 3 mm in relaxed supine position. Both right F) and left H) pampiniform plexus diameter increased higher than 3 mm during Valsava maneuver

condition was taken from the highest grade between right and left varicoceles. About 67.1% of patients presented normal morphology, and 61.2% of the subjects were oligospermic. According to sperm motility, 51.8% of patients were found asthenozoospermia (Table 1).

On statistical analysis, we divided varicocele grade into three groups. There were grade 0 (Figure 1. A-D), grade I (Figure 1. E-H) – grade II (Figure 2. A-D), and grade III (Figure 2. E-F). Dilatation of right, left, and maximum pampiriform plexus with sperm morphology gave p<0.001 (Table 2). Dilatation of right pampiriform with sperm concentration gave p=0.011, dilatation of left and maximum pampiriform p=0.000 (Table 3). Concerning dilatation of right pampiriform and sperm motility we obtained p=0.001, dilatation of left and maximum pampiriform p<0.001 (Table 4). We found significant association between the grade of right varicocele and morphology (p<0.05). The significant association was also found in the grade of left varicocele and the maximum condition (p<0.05).

Table 1. Subject characteristic and data distribution

Characteristic	n (%)	Right	Left	Max
Age groups				
<31 y.o.	17 (20.0)			
31–40 y.o.	55 (64.7)			
>41 y.o.	13 (15.3)			
Education level				
High school	6 (7.1)			
Diploma	9 (10.7)			
Bachelor's degree	44 (51.8)			
Unknown	26 (30.6)			
Occupation status				
Officer	11 (12.9)			
Military	4 (4.7)			
Private employee	49 (57.6)			
Entrepreneur	13 (15.3)			
Unknown	8 (9.4)			
Sperm morphology				
Normozoospermia	57 (67.10)			
Teratozoospermia	17 (20)			
Azoozoospermia	11 (12.9)			
Sperm concentration				
Normozoospermia	22 (25.90)			
Oligozoospermia	52 (61.20)			
Azoospermia	11 (12.90)			
Sperm motility				
Normozoospermia	30 (35.30)			
Asthenozoospermia	44 (51.80)			
Azoospermia	11 (12.90)			
Varicocele grade				
Grade 0		13 (15.3)	8 (9.4)	3 (3.5)
Grade I		9 (10.6)	11 (12.9)	7 (8.2)
Grade II		37 (43.5)	31 (36.5)	29 (34.1)
Grade III		26 (30.6)	35 (41.2)	46 (54.1)

Max= maximum condition, varicocele grade of either left or right testis with largest diameter of pampiniform plexus

Table 2. Grading varicocele and sperm morphology. All parameters were not statistically significant p>0.005

Dilatation of pampiniform plexus	Sperm morphology			n	p
	Normozoo-spermia	Teratozoo-spermia	Azoo-spermia		
Right					<0.001
Grade 0	12	0	1	13	
Grade I-II	28	12	6	46	
Grade III	17	5	4	26	
Left					<0.001
Grade 0	7	0	1	8	
Grade I-II	27	9	6	42	
Grade III	23	8	4	35	
Max					<0.001
Grade 0	2	0	1	3	
Grade I-II	24	7	5	36	
Grade III	31	10	5	46	

Max= maximum condition, varicocele grade of either left or right testis with largest diameter of pampiniform plexus

Table 3. Grading varicocele and concentration

Dilatation of pampiniform plexus	Sperm concentration			n	p
	Normozoo-spermia	Oligozoo-spermia	Azoo-spermia		
Right					0.011
Grade 0	3	9	1	13	
Grade I-II	13	27	6	46	
Grade III	6	16	4	26	
Left					<0.001
Grade 0	4	3	1	8	
Grade I-II	9	27	6	42	
Grade III	9	22	4	35	
Max					<0.001
Grade 0	1	1	1	3	
Grade I-II	9	22	5	36	
Grade III	12	29	5	46	

Max= maximum condition, varicocele grade of either left or right testis with largest diameter of pampiniform plexus

Table 4. Grading varicocele and motility

Dilatation of pampiniform plexus	Sperm concentration			n	p
	Normozoo-spermia	Oligozoo-spermia	Azoo-spermia		
Right					0.01
Grade 0	4	8	1	13	
Grade I-II	19	21	6	46	
Grade III	7	15	4	26	
Left					<0.001
Grade 0	4	3	1	8	
Grade I-II	14	22	6	42	
Grade III	12	19	4	35	
Max					<0.001
Grade 0	2	0	1	3	
Grade I-II	13	18	5	36	
Grade III	15	26	5	46	

Max= maximum condition, varicocele grade of either left or right testis with largest diameter of pampiniform plexus

DISCUSSION

Varicocele is a condition of spermatic vein dilatation in the pampiniform plexus. It is reported that varicocele is associated with infertility.[1] Approximately 8% of men in the reproductive age have infertility problems[8] and 35% of them are caused by varicocele.[4] The previous study found that patient with varicocele did not have Sertoli cell. the Sertoli cell, which function to stimulate follicle stimulating hormone (FSH) and responsible for spermatogenesis.[8,9]

The number of patients with left-sided varicocele is more than the population with right-sided varicocele. This is consistent with the other studies that showed that majority of primary varicoceles were present on the left side. Hasan et al [10] reported that 86.3% of varicoceles were on the left side, whereas Abbas et al[11] revealed that 82% of varicoceles were mainly left-sided. Other studies[1,12] suggested that 78–93% of primary varicoceles were left-sided. The main cause is the anatomical location of left spermatic vein which is more vertical than the right side entering renal vein. Moreover, there is "nutcracker" phenomenon that happened due to compression of the left renal vein, which was located between abdominal aorta and superior mesenterica artery.

The term varicocele grade 0 in this study was defined when the diameter of pampiniform plexus on the Valsalva and non-Valsalva maneuver was less than 2 mm. It's been determined that there was no dilatation of pampiniform plexus. This definition was supported by Pilatz et al[6] who reported that varicocele was the dilatation of pampiniform plexus about 2.45 mm in the relax position (sensitivity 84%; specificity 81%) and about 2.95 mm on the maneuver (sensitivity 84%; specificity 84%) using Doppler ultrasonography.

According to the results of the semen analysis, the majority of sperm morphology was good. However, the sperm concentration and motility were mostly poor. This study is consistent with the semen parameters were influenced by biological, physical, occupational, and environment factor two months before semen analysis examination. Patients was instructed to abstinent for sexually intercourse during 2 until 7 days. Duration of abstinence would influence volume and sperm concentration, but not with sperm motility.[8] The multiple semen analysis disturbance would increase infertility risk. Defect in the sperm morphology was the most crucial factor in the semen analysis, but the result should be interpreted in context of their clinical condition.[8]

This study suggested that varicocele grade is strongly associated with sperm morphology, concentration, and sperm motility. It is caused by increased hidrostatic pressure inside scrotum due to dilatation of pampiniform plexus. This condition increases the temperature, and if the temperature rises, the volume and quality of sperm will be reduced. The lower quality of sperm will impact in the fertilization then. Nevertheless, reference limits provided by WHO manual are derived from semen samples used to initiate natural conception but not the nature of the treatment. Therefore, according to Esteve et al[8] it should be considered to perform additional test, such as genetic evaluation, radiology evaluation, and antibody evaluation.

Patients selected based on inclusion and exclusion criteria. However, there might be several factors that influence the results of this study, such as the past-medical history (cryptorchidism history, herniation, testis trauma, mumps, diabetes mellitus, chirrosis, hypertension, sexually transmitted diseases, tuberculosis and viral infection), surgical history (orchidopexy, herniorraphy, orchiectomy, and surgical at the organ around pelvis), history of gonadotoxic exposure, family history of fibrotic cyst, metabolic endocrine disease and infertility history in family, infertility history (contraception method, pregnancy, and age of wife), and sexual history (frequency of coitus, lubricant used, and libido). Furthermore, larger numbers of the patients is still needed for the better study.[1,4,8]

In conclusion, varicocele grade may affect semen analysis component. A precise medical history, physical examination, and complementary test are the key to establish early diagnosis of varicocele and to determine best strategies to treat infertility.

Conflicts of Interest

The authors affirm no conflict of interest in this study.

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Community Research

The burden of ocular diseases in an underdeveloped village in Southwest Sumba, Eastern Indonesia, 2016

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ABSTRAK

Latar belakang: *Angka prevalensi penyakit mata di suatu daerah penting dalam menentukan rencana program saran kesehatan mata. Tujuan penelitian ini adalah mendeskripsikan beban penyakit mata sebagai langkah awal dalam meningkatkan pelayanan kesehatan mata di daerah yang kurang berkembang di Indonesia.*

Metode: *Sebuah studi potong lintang dilakukan pada warga Desa Perobatang, Sumba Barat Daya (SBD) pada Juli 2016. Pemeriksaan mata dilakukan oleh dokter spesialis mata dan pengukuran tajam penglihatan oleh optometris. Partisipan diberikan pengobatan sesuai dengan diagnosis. Bagi yang membutuhkan, diberikan tindakan operasi dua bulan setelah pemeriksaan.*

Hasil: *Setelah dilakukan pemeriksaan mata terhadap 667 dari total 1.469 penduduk, kelainan mata yang paling sering ditemui adalah presbiopia (30,8%), katarak (12,8%), kelainan refraksi (11,3%), dan pterigium (10,7%). Proporsi miopia sebanyak 5,9%, hiperopia sebanyak 5%, dan astigmatisme sebanyak 2,2%. Proporsi kebutaan ditemukan sebanyak 10%. Katarak menyebabkan kebutaan pada 44 partisipan. Penyebab kebutaan lain yang ditemukan adalah* age-related macular degeneration*, retinopati, atrofi optik, glaukoma,* retinal detachment*, dan trauma.*

Kesimpulan: *Beban penyakit mata di Desa Perobatang, SBD, Indonesia Timur termasuk dalam kategori tinggi. Hasil ini menekankan pentingnya tindakan yang menyangkut kesehatan mata masyarakat dari pemerintah lokal dan lembaga swadaya masyarakat untuk meningkatkan pelayanan kesehatan mata di SBD.*

ABSTRACT

Background: Prevalence estimates of ocular diseases in a given district are important to plan the programs of eye care services. This study aimed to describe the burden of ocular diseases as an initial step in improving eye care services in underdeveloped areas in Indonesia.

Methods: A cross-sectional study was performed among residents of Perobatang Village in Southwest Sumba district in July 2016. Eye examinations were conducted by ophthalmologists, and visual acuity was measured by optometrists. Participants were provided with appropriate treatment according to diagnosis. Surgical services were offered two months after the examination.

Results: After eximining a total of 667 of 1,459 (46%) residents, the result showed that the most frequent ocular problems were presbyopia (30.8%), cataract (12.8%), refractive error (11.3%), and pterygium (10.7%). The proportion of myopia was 5.9%, hyperopia was 5.0%, and astigmatism was 2.2%. Moreover, the proportion of blindness was 10%. Cataract caused blindness in 44 participants. Other causes of blindness included age-related macular degeneration, retinopathy, optic atrophy, glaucoma, retinal detachment and trauma.

Conclusion: The burden of ocular problems in Perobatang Village, Southwest Sumba, Eastern Indonesia was high. These findings showed the importance of public health action from local government and non-governmental organizations to improve eye care services in Southwest Sumba district.

Keywords: blindness, Indonesia, Southwest Sumba, underdeveloped district, visual impairment

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Visual impairment and blindness are major concerns in underdeveloped regions worldwide.[1] Low socio-economic status, lack of awareness, and limited access to eye care services increase the risk of blindness.[2] World Health Organization (WHO) has launched the Universal Eye Health Global Action Plan 2014–2019, which aims to reduce avoidable blindness by 25% in 2020.[3] To this end, WHO promotes the assessment of magnitude and causes of visual impairment by implementing eye care services and encourages the development and implementation of integrated national eye health policies.

As Indonesia is the fourth most populous country in the world, these challenges are very relevant. The overall prevalence of blindness in the general population of Indonesia was 0.6% in 2013.[4] One of the highest blindness rates has been reported in the province of Nusa Tenggara Timur (NTT), at 1% for blindness and 1.6% for low vision.[4] However, due to the vast coverage area and difficult access to remote areas, eye care programs have not been able to reach all areas, including Southwest Sumba district.

Prevalence estimates of ocular diseases in a given district are important to plan the programs of eye care services. Field surveys are required in each district because they may differ substantially in ethnicity, geography, lifestyle, and environmental exposure, making each district unique. Until now, no field survey has been conducted in Southwest Sumba, and the only available data are from the report of the district hospital. The report showed that from 1,363 patients, cataract was the most frequently diagnosed (30.6%), followed by presbyopia (15.6%), myopia (9%), conjunctivitis (6.7%), pterygium (4.3%), and glaucoma (2.6%).[5] The eye examinations and treatment, however, were performed by trained nurses rather than an ophthalmologist.

Based on the challenges above, we aimed to perform a field survey in Southwest Sumba District to estimate the local burden of ocular diseases. Such baseline information was envisaged to help shape programmatic planning of eye care services in the area. Perobatang Village was selected because of its impoverished condition, lack of access to health care, lack of clean water, and low hygienic practices. Thus, it represents the most underdeveloped districts in Indonesia.

METHODS

Study area

Perobatang Village is a small rural community located 70 km from the capital of South West Sumba district, Tambolaka. It has an area of 4,360 hectares with 315 families and a total population of 1,459 people, with the following age distribution: 498 people of 0–18 years, 231 people of 19–39 years, and 630 people of >40 years. The predominant occupations are fishermen and farmers.

There is no primary health care (PHC) facility in the community, and the nearest PHC is located about 3 km away in Bondokodi subdistrict. The nearest public eye care facility is in Karitas Hospital located in Tambolaka. Access to clean water is limited to public mono-pumps. Open defecation is still practiced in the bush, and only a few households have latrines, thus the prevalence of helminthiases is high. Study conducted in 2016 showed the prevalence of soil transmitted helminthiases was 76%, consisting of *A. lumbricoides* (60%), *T. trichiura* (64%), and *hookworm* (10%).[6] Such conditions reflect the poor sanitation and hygiene of Perobatang residents.

Data Collection

The cross-sectional survey among village residents was conducted in July 2016 at the village chief's house, which was transformed into a makeshift clinic. The study population included all village residents, who were actively approached to undergo the eye examination by the village chief. All residents with or without ophthalmic complaints were asked to participate in the study.

The medical team consisted of five ophthalmologists, two optometrists, three ophthalmology residents, and four medical students from Faculty of Medicine, Universitas Indonesia. After oral inform consent was obtained, medical history was taken, followed by eye examinations. Visual acuity was examined using an auto-refraction machine and Snellen chart. Vision of 6/60 was assessed by counting fingers at less than six meters; if failed a flashlight was used to assess light perception.

Visual acuity was classified as normal (≥6/18), low (<6/18–3/60), and blindness (<3/60).

Children who were unable to read or pointed out directions were examined with the fix and follow test. Improvements of vision after a pinhole test were categorized as refractive errors and given a prescription for corrective lenses, provided two months after the examination.

An anterior segment evaluation was done using a hand slit-lamp, and funduscopy was performed if indicated, with pupils being dilated using tropicamide 1% eye drops. A posterior segment assessment was carried out using a direct ophthalmoscope, and intraocular pressure was measured using Schiotz tonometer.

Diagnosis was conducted by ophthalmologists, and subjects were treated based on their diagnosis in accordance with clinical standards. Eye infections were treated with antibiotics and dry eye were treated with artificial tear drops. Refractive errors were corrected with spectacles, and patients with cataract, pterygium and glaucoma were scheduled for surgeries in Karitas Hospital two months after the examination. The surgery was carried out using the phacoemulsification technique or extra-capsular cataract extraction for patients with hard cataracts or weak epithelial tissue in the cornea. Ethical approval was obtained from the ethical committee of Faculty of Medicine, Universitas Indonesia (clearance number 877/UN2.F1/ETIK/2016 protocol number 16281).

RESULTS

Demographic characteristics of the subjects

From 1,459 residents of Perobatang, 667 people voluntarily enrolled to get eye examination (46%). Table 1 showed that most of the subjects were <40 years (56.8%), women (58.8%) and primary school graduates (37.9%). The age ranged from 4 months to 80 years. Occupations included students (27.6), fishermen (25.5), farmers (19.9%), housewives (21.7%) and others (5.3%).

The proportion of ocular problems

The number of subjects for one ocular disease was the total subjects having ocular disease in left eye, right eye or both eyes as shown in Table 2. One subject may have more than one ocular disease. The most frequently observed ocular problems were presbyopia (30.8%), cataract (12.8%), refractive error (11.3%) and pterygium (10.7%). Former trauma cases were found in the form of corneal cicatrix (1.1%) and traumatic cataract (0.7%). There were 67 subjects with blindness (10%). Degenerative diseases contributed in causing blindness to Perobatang residents, such as age-related macular degeneration, retinopathy, and optic atrophy.

Table 1. The demographic characteristics of Subjects in Perobatang Village, 2016

Characteristics	Total n (%)	95% CI
Gender		
Male	275 (41.2)	37.5–44.9
Female	392 (58.8)	55.1–62.5
Age		
0–16	204 (30.6)	27.1–34.1
17–39	175 (26.2)	22.9–29.5
≥40	288 (43.2)	39.4–47.0
Education		
No education	82 (12.3)	9.8–14.8
Primary school	253 (37.9)	34.2–41.6
Junior high school	104 (15.6)	12.9–18.4
Senior high school	175 (26.2)	22.9–29.5
Undergraduate	53 (8.0)	5.9–10.1
Occupation		
Students	184 (27.6)	24.2–31.0
Fisherman	170 (25.5)	22.2–28.8
Housewife	145 (21.7)	18.6–24.8
Farmer	133 (19.9)	16.9–22.9
Others	35 (5.3)	3.6–7.0

Small numbers of ocular diseases were also found such as conjunctivitis, dry eye, retinopathy, amblyopia, glaucoma, subconjunctival hemorrhage, entropion, corneal cicatrix, pingueculitis, optic atrophy, retinal detachment, phthisis bulbi, adherent leukoma and uveal prolapse.

To identify the proportion of ocular problems in children (0–16 year-age group), Table 3 shows the proportion of ocular problems among 204 participants. The most common ocular problems were refractive error (2%) and blindness (2.5%); most blindness was due to trauma (5%). Other ocular problems were conjunctivitis, intermittent strabismus, subconjunctival hemorrhage and optic atrophy.

Table 2. The proportion of ocular problems among residents of Perobatang Village, 2016 (n=667)

Diagnosis	OD n (%)	OS n (%)	ODS n (%)	Subjects n (%)
Ocular Problems*				
Presbyopia	–	–	–	206(30.8)
Cataract	4(0.5)	9(1.3)	73(10.9)	86(12.8)
Refractive error	6(0.8)	8(1.1)	62(9.2)	76(11.3)
Pterygium	19(2.8)	15(2.2)	38(5.6)	72(10.7)
Pseudophakia	6(0.8)	1(0.1)	1(0.1)	8(1.1)
Conjunctivitis	0	1(0.1)	3(0.4)	4(0.5)
Dry eye	1(0.1)	1(0.1)	2(0.2)	4(0.5)
Aphakia	0	1(0.1)	2(0.2)	3(0.4)
Retinopathy	0	1(0.1)	2(0.2)	3(0.4)
Amblyopia	0	1(0.1)	1(0.1)	2(0.2)
Lithiasis	1(0.1)	1(0.1)	0	2(0.2)
Strabismus intermittent	0	0	1(0.1)	1(0.1)
Glaucoma	0	1(0.1)	0	1(0.1)
Subconjunctival hemorrhage	1(0.1)	0	0	1(0.1)
Entropion	1(0.1)	0	0	1(0.1)
Pingueculitis	1(0.1)	0	0	1(0.1)
Blindness				
Cataract:	9(1.3)	14(2.0)	21(3.1)	44(6.5)
Cataract	6(0.8)	13(1.9)	18(2.6)	37(5.5)
Cataract + refractive error	1(0.1)	0	2(0.2)	3(0.4)
Cataract + corneal cicatrix	1(0.1)	0	0	1(0.1)
Cataract + AMD	1(0.1)	0	0	1(0.1)
Cataract + retinopathy	0	0	1(0.1)	1(0.1)
Cataract + retinal detachment	0	1(0.1)	0	1(0.1)
Corneal cicatrix	3(0.4)	2(0.2)	2(0.2)	7(1.0)
Optic atrophy	2(0.2)	1(0.1)	1(0.1)	4(0.5)
Retinal detachment	1(0.1)	1(0.1)	0	2(0.2)
Aphakia	0	0	2(0.2)	2(0.2)
Refractive error	2(0.2)	1(0.1)	2(0.2)	2(0.2)
Amblyopia	0	0	1(0.1)	1(0.1)
Phthisis bulbi	1(0.1)	0	0	1(0.1)
Anophthalmia	1(0.1)	0	0	1(0.1)
Nonfunctional eye	1(0.1)	0	0	1(0.1)
Adherent leukoma	0	1(0.1)	0	1(0.1)
Uveal prolapse	0	1(0.1)	0	1(0.1)
Total blindness				67 (10)

*One subject may have more than one ocular disease. OD= oculi dextra; OS= oculi sinistra, ODS= oculi dextra et sinistra; AMD=age-related macular degeneration

Refractive Error

The proportion of myopia was 5.9%, hyperopia 5.0% and astigmatism was 2.2% (Table 4). There were two subjects with myopia and one subject with hyperopia who had high difference of refractive power (more than 2.5 dioptri between both eyes).

As shown in Table 5, myopia and hyperopia were mostly seen in subjects >40 years old, primary

Table 3. Proportion of ocular problems among children 0–16 years old, Perobatang Village, 2016 (n=204)

Diagnosis	OD n (%)	OS n (%)	ODS n (%)	Subjects n (%)
Ocular Problems				
Refractive error	0	1 (0.4)	3 (1.4)	4 (1.9)
Conjunctivitis	0	1 (0.4)	2 (0.9)	3 (1.4)
Strabismus intermittent	0	0	1 (0.4)	1 (0.4)
Subconjunctival hemorrhage	1 (0.4)	0	0	1 (0.4)
Blindness				
Corneal cicatrix (trauma)	0	1 (0.4)	0	1 (0.4)
Traumatic cataract	1	0	0	1 (0.4)
Traumatic cataract + corneal cicatrix	1 (0.4)	0	0	1 (0.4)
Optic atrophy	0	0	1 (0.4)	1 (0.4)
Uveal prolapse (trauma)				1 (0.4)
Total blindness	0	1 (0.4)	0	5 (2.5)

Table 4. Proportion of refractive error among residents in Perobatang Village, 2016

Refractive error	OD n (%)	OS n (%)	ODS n (%)	Subjects n (%)	Δ2.5D
Myopia	4 (0.5)	8 (1.1)	28 (4.1)	40 (5.9)	2 (0.2)*
Hyperopia	3 (0.4)	2 (0.2)	29 (4.3)	34 (5.0)	1 (0.1)†
Astigmatism	3 (0.4)	3 (0.4)	9 (1.3)	15 (2.2)	–
Simple astigmatism	0	1 (0.1)	1 (0.1)	2 (0.2)	–
Astimagtism + myopia	0	0	5 (0.7)	9 (1.3)	–
Astimagtism + hyperopia	0	1 (0.1)	3 (0.4)	4 (0.5)	–

*female 26 years old, female 59 years old. †female 69 years old.One subject may have more than one type of refractive error

school graduates, and farmers. Hyperopia was more common in female (3.2%) than myopia (2.9%).

The proportion of cataract

Cataract was diagnosed in 93 subjects (13.1%) (Table 6). Cataract caused blindness in 44 (47.3%) subjects, 21 (47.7%) of which suffered from bilateral blindness. There were 33 (35.5%) subjects who had cataract surgery indications and scheduled for surgery within two months after the examination.

DISCUSSION

The goal of the 2014–2019 Universal Eye Health Global Action Plan, which was encouraged by WHO, is to reduce avoidable blindness by 25% in 2019. This is essential to plan further eye care services and problem-solving measures by nations worldwide. WHO expects active participation from the government and other stakeholders to implement national policies and plans to eliminate blindness. National policy is expected to be carried out nation-wide from primary care level to referral centers. These steps have been taken by nations worldwide, including Indonesia. However, Indonesia experiences difficulties due to its challenging geographical access and vast area coverage leaving rural, remote, and underdeveloped districts unattended.

As an initial step, collection of data regarding pattern and prevalence of ocular diseases is essential especially in rural and underdeveloped areas. Perobatang Village is an excellent example as it highly requires medical assistance. This study showed that the most common ocular problems at Perobatang Village, Southwest Sumba

Table 5. Proportion of refractive error based on demographic characteristics of the subjects

Characteristics	Myopia n (%)	Myopia + ast n (%)	Hyperopia n (%)	Hyperopia + ast n (%)	All ast n (%)	Simple ast n (%)
Age						
0–16	2 (0.2)	0	1 (0.1)	0	1 (0.1)	1 (0.1)
17–39	13 (1.9)	3 (0.4)	1 (0.1)	1 (0.1)	4 (0.5)	0
≥40	25 (3.7)	6 (0.8)	32 (4.7)	3 (0.4)	10 (1.4)	1 (0.1)
Gende						
Male	20 (2.9)	5 (0.7)	12 (1.7)	1 (0.1)	7 (1.0)	1 (0.1)
Female	20 (2.9)	4 (0.5)	22 (3.2)	3 (0.4)	8 (1.1)	1 (0.1)
Education						
No education	2 (0.2)	1 (0.1)	0	0	1 (0.1)	0
Primary school	14 (2.0)	3 (0.4)	15 (2.2)	3 (0.4)	7 (1.0)	1 (0.1)
Junior high school	5 (0.7)	2 (0.2)	2 (0.2)	1 (0.1)	3 (0.4)	0
Senior high school	13 (1.9)	0	16 (2.3)	0	1 (0.1)	1 (0.1)
Undergraduate	6 (0.8)	3 (0.4)	1 (0.1)	0	3 (0.4)	0
Occupation						
Fisherman	6 (0.8)	1 (0.1)	5 (0.7)	1 (0.1)	2 (0.2)	0
Farmer	15 (2.2)	3 (0.4)	9 (1.3)	1 (0.1)	4 (0.5)	0
Other	19 (2.8)	5 (0.7)	20 (2.9)	2 (0.2)	9 (1.3)	2 (0.2)

Ast= astigmatism

Table 6. Proportion of cataract among residents in Perobatang Village, 2016

Type	OD n (%)	OS n (%)	ODS n (%)	Subjects n (%)
Immature	6 (0.8)	6 (0.8)	60 (8.9)	72 (10.7)
Mature	2 (0.2)	6 (0.8)	6 (0.8)	14 (2.0)
Hypermature	1 (0.1)	0	0	1 (0.1)
Traumatic	2 (0.2)	3 (0.4)	0	5 (0.7)
Complicated	0	1 (0.1)	0	1 (0.1)

OD= ocular dextra; OS= ocular sinistra; ODS= ocular dextra and sinistra

were presbyopia, refractive error, cataract, and pterygium. Blindness and visual impairment in the districts were high as they were lack of health facilities especially eye care services. These districts also suffered from poverty and low education, enhancing the magnitude of the problem.

Presbyopia is an age-related eye disorder caused by progressive loss of accommodative amplitude that is still a major concern in developing countries. Man et al[7] reported high (33.9%) prevalence of uncorrected presbyopia in a multiethnic Asian population. Nearly all patients with presbyopia did not possess spectacles, and those subjects had difficulties in daily activities.[8] Study in East Africa found that only 18% of 340 patients suffering from presbyopia had spectacles because they could not afford spectacles, the insubstantial need of spectacles, and the lack of access.[9,10] Nirmalan et al[11] reported the noticeable difficulties in activities requiring near-vision; patients complaint of difficulties in recognizing small objects and "unable to manage any near world". This study found high proportion of presbyopia (30.8%). This condition was worsened by lacking of corrective glasses which led to difficulties in weaving traditional fabrics as one of their primary sources of income. In addition, they also had difficulties in reading small letters such as those in Qur'an, Bible, or newspapers.[12]

Refractive error is the most common etiology of visual impairment due to failure of precisely focusing rays of light from an object onto the retinal layer resulting in blurred vision and requiring a refractive correction in order to see clearly.[11–12] In this study, there were 76 participants (11.3%) with refractive errors, four of them were in 0–16 year age group. This condition required refractive

correction as it caused moderate to severe visual impairment. In addition, there were two subjects with myopia and one subject with hyperopia who had high difference of refractive power (more than 2.5 dioptri between both eyes). Difference of more than 2.5 dioptri between two eyes require contact lens or refractive surgery, in which both are inaccessible to these participants. In this study, myopia was more common in male; however, most studies showed no association between gender and hyperopia.[14] Most people with refractive error were farmers and fishermen. Outdoor activity has been associated with refractive error development. People with limited outdoor time and high usage of eye accommodation (i.e tailor and waiver) tend to be myopic; on the other hand, those with high levels of outdoor activity had the most hyperopic mean refraction.[15]

Uncorrected refractive error was the leading cause of moderate to severe visual impairment. Hence, it requires appropriate refractive correction, using spectacles, contact lenses, or refractive surgery. Spectacles are mostly used as treatment of corrective error in developing countries because of its simplicity and affordable cost. Residents of Perobatang Village who had their eyes examined and were diagnosed with refractive error, had not used spectacles. Most of them could not afford spectacles, thus we provided spectacles two months after examination. The two subjects with myopia and one subject with hyperopia who had high difference of refractive power require referral to cities with eye care centers to receive corrective contact lenses or refractive surgery.

Cataract is defined as lens opacification with blurred vision and experiencing glare or haloes from lights as the symptoms.[16] Although aging is the most well-known risk factor for cataract development, there are other individual factors such as socioeconomic status, gender, certain racial or ethnic groups, and genetic factor. Lifestyle could affect cataract development, which are ultraviolet-B exposure, cigarette smoking, and alcohol consumption. Other factors in cataract formation are diet, systemic diseases, and ocular disorder.[17] People living in Perobatang Village mostly work as fishermen or farmers. They spend most of their working hours outdoor without sun-glasses or other protective measures, hence they are heavily exposed to sunlight. The proportion of cataract in our study was 16.1%, while Indonesian National Health Survey in 2013 showed that the cataract prevalence was 1.8%, with the highest prevalence in North Celebes (3.7%) and the lowest was in Jakarta (0.9%).[4] The National Survey also showed that most cataract patients were not aware of the presence of cataract (51.8%) and were reluctant to undergo surgery due to financial problems (11.6%), fear of surgery (8.1%), and belief that cataract was a normal aging process.[4,17] Most of cataract patients in Perobatang Village were not reluctant to undergo surgery, however due to lack of funding for transportation and hospital fee, they could not undergo surgery and in hope to the social service for cataract operation. In our study, there were 44 participants suffering from blindness due to cataract, 21 of them had bilateral blindness; such cases must be prioritized for surgery. If one of the vision is corrected through the surgery, the patient could have a normal life.

Pterygium is a fibrovascular proliferative disease of bulbar conjunctiva towards cornea with irritation, visual disturbances, cosmetic issues as symptoms, and highly associated to ultraviolet (UV) exposure.[18,19] In Perobatang Village, the proportion of pterygium was 10.7% while the Indonesian Health Survey in 2013 showed the prevalence of pterygium was 8.3% with the highest was in Bali (25.2%) and the lowest was in Banten (3.9%).[4] The prevalence of pterygium was higher in lower latitudes, which has higher sunlight exposure.[20] Another risk factor was the cumulative time spent doing outdoor activities. Public education to encourage the use of appropriate protective measures for outdoor workers is very important. It is recommended to wear sunglasses and hats to minimize exposure to UV and strong wind.

This study provides a glimpse of the public health challenges that are faced by developing countries. There are many underdeveloped regions with undiagnosed ocular diseases requiring specific programs and support from the government through their policies. The absence of eye care services in this underdeveloped community contribute to the high prevalence of blindness and visual impairment. Eye disorders are not detected and managed in a timely manner, allowing them to deteriorate and eventually progress to blindness. The only hospital in the district does not offer any eye care services by ophthalmologists. Given the

long distance, logistics, and costs, only few of the villagers could afford to seek eye care services located in other islands.

Currently, there are only six ophthalmologists in NTT Province serving one vast province with many islands which are difficult to access. Thus, the allocation of the regional budget expenditure to recruit more ophthalmologists is required. These visitations must be supported by government, non-governmental organizations (NGOs), universities, professional organizations, and other stakeholders in terms of mobile facilities, other logistics, and financing to be sustainable. The event must take place locally and easy to access as people who live in rural area tend to be reluctant to seek health facilities and would rather to be treated in their residents. Other solution is working together with nearby medical schools to send their chief ophthalmology residents to remote areas as part of their training program.

Furthermore, local government must participate actively by announcing the schedule of the event to people and organizing it.[21] This, in hope with improvement of local health facilities, will go a long way in improving vision of people in underdeveloped regions and their quality of life. This must be carried out not only in Southwest Sumba, but also in other underdeveloped districts where shortage of manpower and facilities exist. Southwest Sumba district is one of the magnets of tourism in the eastern part of Indonesia. The tourism industry in this region could help in term of giving back to the people by conducting a mass social service in eye care program as part of the community social responsibility programs.

The limitation of this study was the cross sectional study with no follow up data. However, this study serves as an important reminder for government officials to start prioritizing programs to bring eye care to remote areas as suggested by WHO by reallocating their funds and channel their concerns. This survey serves as an example not only to the Indonesian government, but also to other developing nations with challenging geographical terrains and unequal distribution of resources. This survey highlights the importance of eye care in remote underdeveloped areas and urges the government to support periodic annual visitations by ophthalmologists to conduct eye examinations and surgeries.

In conclusion, the burden of ocular problems in Perobatang Village, Southwest Sumba, Eastern Indonesia was high. The variety of ocular problems such as presbyopia, refractive error, cataract, and pterygium could be a based on priority for government program in Southwest Sumba district.

Conflicts of Interest

Saleha Sungkar is one of the editorial board members, but was not involved in the review or decision process of the article.

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Community Research

Association between parental socio-demographic factors and declined linear growth of young children in Jakarta

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ABSTRAK

Latar belakang: *Di Indonesia, diperkirakan 35,5% anak berusia kurang dari lima tahun memiliki perawakan pendek. Perawakan pendek berkaitan dengan postur pendek saat dewasa, kemampuan kognitif dan kecerdasan yang rendah, penghasilan yang rendah, hilangnya produktivitas dan risiko penyakit kronis terkait nutrisi. Penelitian ini bertujuan untuk mengetahui pengaruh faktor sosiodemografi orang tua terhadap penurunan pertumbuhan linear pada anak usia kurang dari dua tahun.*

Metode: *Penelitian dilakukan secara kohort prospektif sejak Agustus 2012 hingga Mei 2014 pada tiga pusat kesehatan masyarakat (Puskesmas) di wilayah Jakarta, Indonesia, yaitu Puskesmas Kecamatan Jatinegara, Mampang, dan Tebet. Subjek adalah anak sehat berusia kurang dari 2 tahun, yang dilakukan pengukuran berat badan dan tinggi badan serial (pada usia 6–11 minggu dan usia 18–24 bulan).* Length-for-age *berdasarkan parameter tersebut digunakan untuk menentukan status perawakan. Pengukuran serial bertujuan mendeteksi pola pertumbuhan linier. Data sosio-demografik orangtua didapatkan melalui kuesioner.*

Hasil: *Dari total 160 subjek, 14 (8,7%) anak mengalami penurunan pertumbuhan linier dari normal menjadi* stunted*, dan 10 (6,2%) anak lainnya menurun menjadi* severely stunted*. Sebanyak 134 (83,8%) anak memiliki pola pertumbuhan yang stabil normal. Hanya 2 (1,2%) anak menunjukkan perbaikan pada pertumbuhan liniernya. Durasi pendidikan ibu kurang dari 9 tahun (RR=2,60, 95% IK=1,23–5,46; p=0,02) menunjukkan hubungan bermakna dengan penurunan pertumbuhan linier anak.*

Kesimpulan: *Durasi pendidikan ibu kurang dari 9 tahun merupakan faktor risiko penting terjadinya penurunan pertumbuhan linier pada anak usia kurang dari 2 tahun.*

ABSTRACT

Background: In Indonesia, approximately 35.5% of children under five years old were stunted. Stunting is related to shorter adult stature, poor cognition and educational performance, low adult wages, lost productivity, and higher risk of nutrition-related chronic disease. The aim of this study was to identify parental socio-demographic risk factors of declined linear growth in children younger than 2 years old.

Methods: This was a cohort-prospective study between August 2012 and May 2014 at three primary community health care centers (Puskesmas) in Jakarta, Indonesia, namely Puskesmas Jatinegara, Mampang, and Tebet. Subjects were healthy children under 2 years old, in which their weight and height were measured serially (at 6–11 weeks old and 18–24 months old). The length-for-age based on those data was used to determine stature status. The serial measurement was done to detect growth pattern. Parental socio-demographic data were obtained from questionnaires

Results: From the total of 160 subjects, 14 (8.7%) showed declined growth pattern from normal to stunted and 10 (6.2%) to severely stunted. As many as 134 (83.8%) subjects showed consistent normal growth pattern. Only 2 (1.2%) showed improvement in the linear growth. Maternal education duration less than 9 years (RR=2.60, 95% CI=1.23–5.46; p=0.02) showed statistically significant association with declined linear growth in children.

Conclusion: Mother with education duration less than 9 years was the determining socio-demographic risk factor that contributed to the declined linear growth in children less than 2 years of age.

Keywords: children, maternal education, risk factor, socio-demographic, stunting

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Attained height is affected by genetic and environmental factors throughout the growth period. Any problems in one of those factors may lead to stunting which is defined as length/height-for-age below -2 SD of the World Health Organization (WHO) child growth standards median.[1] Stunting is the result of inadequate nutritional intake starting from the period of conception to 24 months old.[2,3] Stunting in the first 2 years will remain until adulthood; it contributes to shorter adult stature[1] and is associated with other consequences, such as poor cognition and educational performance, low adult wages, lost productivity, and higher risk of nutrition-related chronic disease.[2,3]

Stunting is a global burden because 80% of them occur in developing countries. Southeast Asia has the second highest prevalence, and total number of children stunted are 15.6 million (29.4%). Indonesia belongs to the top 20 countries with the highest burden of malnutrition which constitutes 80% of the world's undernourished children.[4] Based on the 2010 United Nation of Children Education Fund (UNICEF) data, prevalence of stunting in Indonesia was reported to be approximately 35.5% of all children.[3]

Child stunting is associated with constitutional factors (e.g. maternal height, birth weight), risk factors (e.g. nutritional intake, illness), and external factors (e.g. parental education, socio-economic status, poverty). Some of external factors such as low parental education has been reported from Semarang,[5] mother's education of 24–59 months old children reported from North Sumatera province;[6] parental education, family economic status has been reported from Depok,[7] but there was lack of published report from Jakarta. Jakarta as the capital city of Indonesia is the most populous city in Indonesia and has a wide variation in socio-demographic background which might influence the rearing practice and eventually affects the linear growth of children. This study aimed to identify parental socio-demographic risk factors of declined linear growth in children younger than 2 years old in Jakarta.

METHODS

This prospective cohort study was conducted from August 2012 to May 2014 in Jatinegara, Tebet, and Mampang Health Centers, Jakarta, Indonesia. Subjects of this study were those of longitudinal vaccine study with one and half year interval follow-up. They were children aged <24 months, clinically healthy, born between 37 and 42 weeks of gestation with birth weight of 2,500–4,000 g. Children with febrile or severe illness or congenital abnormalities or children with routine medical drug consumption were excluded.

This study was approved by the Health Research Ethics Committee, Faculty of Medicine Universitas Indonesia – Cipto Mangunkusumo Hospital (101/PT02.FK/ETIK/2012 and 735/H2.F1/ETIK/2013). Informed consent was acquired from participant parents or guardians.

Demographic and anthropometric measurement

The characteristics of subjects obtained were age, sex, birth order, weight, and length. The parental socio-demographic data were age, highest education level, and occupation of the mother and father. Socio-demographic data were obtained by interview using a guided questionnaire. First measurement was conducted when subjects were 6 to 11 weeks of age, and the second measurement was at 18 to 24 months of age. Body weight was measured with minimal clothing using calibrated baby scale (Seca, Germany) with accuracy of 1 gram. Body length was measured in recumbent position using length board with accuracy of 1 mm. Recumbent length was measured while child was lying down straight along the board, head against the headboard with eyes looking straight up, body and legs straight and flat in the center of the measuring board, heels and feet firmly against the foot board. Measurement was done to the nearest mm. Anthropometric data were plotted to the 2005 WHO growth curve to determine the length-for-age (LFA).

Interpretation of LFA was as follow: (1) normal if LFA z-scores between –2 to +3; (2) stunted if LFA z-score between –2 to –3 z-scores; and (3) severely stunted if LFA z-scores ≤–3 SD. Linear growth was assessed as follow: (1) constant if there was no increment or decrement of LFA z-scores between two measurements; (2) inclined or declined if there was an increment or decrement of LFA which passed –2 z-scores, respectively.

Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences (SPSS) version 21.0.

Dependent variable was pattern of linear growth, which was classified into (1) inclined or constant, and (2) declined. Independent variables were the socio-demographic data of subjects and parents that were classified as follows: subject's birth order (first to second order or third order and up), parental age (20–35 years old or ≥ 35 years old), parental education duration (>9 years (senior high school or diploma/university degree) or ≤9 years (no education, primary – junior high school)), mother employment status (housewife or working), and father occupation (labor, civil servant, private sector, entrepreneur, or others).

Analysis of the association between each independent variable and dependent variable was done by Chi-square or Fisher test as appropriate, with $p<0.05$ considered as statistically significant. For paternal occupation with 5 subgroups, analysis was performed independently with a pattern of linear growth using Chi-square of Fisher test as appropriate. Reference group used in this analysis was the total of non-tested group. Relative risk and 95% confidence interval (CI) were calculated for each independent variable.

RESULTS

There were 258 children who participated in the first measurement, but only 160 of them participated in the second measurement. The 98 children who did not participate in the second measurement had been moved to another area due to natural heavy flood and loss of contact.

The mean and standard deviation of the subjects age were 8.0±1.6 weeks and 19.9±0.8 months in the first and second measurement respectively. High school was the highest level of education for mother (55.6%) and father (58.8%). Most of the mothers were housewives (80%), and the fathers were working in private sector (43.8%) (Tabel 1).

In the first measurement, the LFA classification of 158 (98.8%) subjects were normal and 2 (1.2%) were stunted. In the second measurement, out of 160 subjects, 24 (15%) showed declined LFA; 14 of them (8.7%) declined from normal, crossed –2 z-scores and classified as stunted and the rest 10 (6.2%) crossed –3 z-score and classified as severely stunted. About 134 children (83.8%) had the same normal growth classification, and only 2 children (1.2%) inclined from stunted into normal range (Figure 1).

Univariate analysis of the association between each independent factor and the dependent factor was presented in Table 2. Children with working mothers had about one sixth risk of declined linear growth (RR=0.17, 95% CI=0.02–1.24; p=0.05) as compared to those whose mothers were housewives. (Table 2) Paternal education duration less than 9 years was related to 1.6

Table 1. Subjects characteristics and parents socio-demographic characteristics

Variables	n	%
Sex		
Male	92	57.5
Female	68	42.5
Subject's birth order		
1	66	41.3
2–3	82	51.3
4–6	12	7.5
Mother's age (years)		
<20	3	1.9
20–35	129	80.6
>35	28	17.5
Mother's education level		
No education background	2	1.3
Primary – junior high school	54	33.8
Senior high school	89	55.6
Diploma – Bachelor degree	15	9.4
Mother's occupational status		
Working	32	20
Housewife	128	80
Father's age (years)		
<20	2	1.3
20–35	90	56.3
>35	68	42.5
Father's education level		
No education background	2	1.3
Primary – junior high school	47	29.4
Senior high school	94	58.8
Diploma – Bachelor degree	17	10.6
Father's occupation status		
Labor	17	10.6
Civil servant	2	1.3
Private sector	70	43.8
Entrepreneur	57	35.6
Other	14	8.8

times higher risk of children with declined linear growth (RR=1.61, 95% CI= 0.77–3.38; p=0.23).

Birth order, maternal age, and paternal age were considered not associated significantly

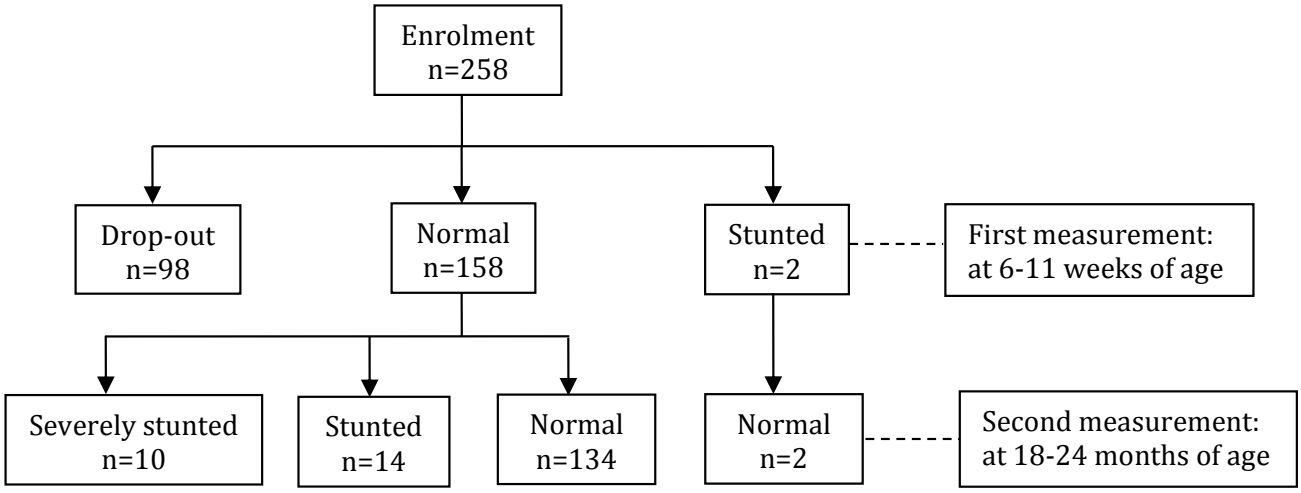

Figure 1. Subjects' linear growth changes

Table 2. Association between socio-demographic factors and subjects' linear growth

	Inclined or constant		Declined		RR	CI 95%	p
	n	%	n	%			
Subject's birth order						0.95–4.18	
1–2	108	87.8	15	12.2			Ref
≥3	28	75.7	9	24.3	1.99		0.11
Maternal age (years)						0.37–2.76	
20-35	29	82.9	6	17.1			Ref
<20 and >35	107	85.6	18	14.4	1.02		0.58
Maternal education duration (years)						1.24–5.47	
>9 years	94	90.4	10	9.6			Ref
≤9 years	42	75.0	14	25	2.60		0.02
Maternal employment status						0.02–1.24	
Housewife	105	82.0	23	18.0			Ref
Working	31	96.9	1	3.1	0.17		0.05
Paternal age (years)						0.53–2.96	
20–35	66	82.5	14	17.5			Ref
<20 and >35	70	87.5	10	12.5	1.25		0.38
Paternal education duration (years)						0.77–3.39	
>9 years	97	87.4	14	12.6			Ref
≤9 years	39	79.6	10	20.4	1.62		0.23
Paternal occupation*							
Labor	13	76.5	4	23.5	0.56	0.17–1.88	0.31
Civil servant	1	50.0	1	50	0.18	0.01–2.96	0.29
Private sector	62	88.6	8	11.4	1.80	0.73–4.47	0.20
Entrepreneur	47	82.5	10	17.5	0.80	0.33–1.92	0.62
Other	12	85.7	2	14.3	1.12	0.23–5.35	1.00

CI= confidence interval; Ref= reference; RR= relative risk; *= Reference was the total of non-tested group as compared to all other occupations

with children linear growth (p>0.05). Maternal education duration (RR=2.60, 95% CI=1.24–5.47; p=0.02) showed statistically significant association with children linear growth. This meant that mother with education duration less than 9 years had 2.6 times higher risk of having children with declined linear growth than mother with education more than 9 years. Independent analysis of father employment status subgroups showed no statistical significant association with children linear growth.

DISCUSSION

Linear growth failure reflects the major public health problem caused by malnutrition. About 165 million of children under five years old were affected, and the highest was found in Southern Asia (56%) and Africa (36%).[8] In this study, we found that 24 out of 160 (15%) subjects with declined linear growth, resulting in stunting and severe stunting. The stunting prevalence in this study was lower than stunting prevalence in Jakarta which was 27.5% as reported by the 2013 basic health survey.[9] This difference might be due to better infant feeding practice, better nutritional intake or better socio-economic factors in our subjects which were not explored further as it was beyond the scope of this study.

In general, children growth is indicated by weight and length/height. Growth failure or failure to thrive was considered if a child's weight is below the 5th percentile, if it drops down more than 2 major percentile lines, or if weight for height is less than 5th percentile.[10] Since we studied the linear growth, we used length as the variable. The advantage of using length is a stable indicator and not influenced by acute condition such as dehydration and it represents long term growth.

Children growth often shifts percentiles for both length-for-age (LFA) and weight-for-age (WFA), especially in the first 6 months. However, majority of shifting is settling into a curve towards the 50th percentile rather than away.[11] The shifting is regarded as normal as long as in normal range of –2 SD to +2 SD. If LFA fall to below -2SD from the median for LFA, the child can be classified as stunting according to the WHO multicenter growth reference study group.[1] Majority of stunting occurred in the first 1,000 days or before 2 years old.[12] Stunting before the age of 2 years old predicts poorer cognitive and educational outcomes in later childhood and adolescence[13] and has significant educational and economic consequences at the individual, household, and community levels. Some catch up is possible in height-for-age after 24 months, with uncertain cognitive gains.[14]

About 24 subjects who had normal LFA at 6–11 weeks of age declined to stunted (14 subjects) and severely stunted (10 subjects) at 18–24 months of age. Changes in growth trajectory were associated with many factors. Several studies have found factors, such as child's gender, age, birth order, parental education, and parental working status to be common determinants of stunting.[15] Studies in Indonesian children reported that factors related to children stunting were lower birth weight, low parental height, low maternal education, higher family member, male sex, living in urban area and low sanitation.[16, 17]

There was no significant association between children birth order, maternal age, and maternal employment status variables with children linear growth in this study. Majority of the subjects 136 out of 160 (85%) had constant or inclined linear growth. From birth order, as many as 15 out of 24 children with declined linear growth were first- or second- born child. The poor association between maternal age and parity with linear growth was also found in a study in South Africa.[18] However, in studies with large sample, there was association noted between birth order and children stunting. A study of 12,830 children in England reported that older mother was associated with higher body length, and higher parity was associated with risk of stunting.[19] In a Bangladesh study, birth order was one of the significant predictors of children stunting. Third order, fourth order, and fifth or higher order children were 24%, 30%, and 72%, respectively, more likely to be stunted.[20] This was possibly due to favoritism toward eldest sons, which affected parents' fertility decisions and resource allocation across children.[21] Birth order as a risk factor of children stunting was noted in studies with huge sample, in which a little difference of clinical characteristics may yield statistically significant result. This contradicting birth order result needs further study to shed light of the association.

Maternal education was correlated significantly with their children linear growth pattern. As much as 94 out of 104 (90.4%) mothers with education for more than 9 years had children with good linear growth in this study. On the other hand, 14 out of 24 (58.3%) subjects with declined linear growth had mothers with education duration less than 9 year. The higher the maternal level of education, the better the linear growth. This result was consistent with the study conducted in Indonesia, Bangladesh and Uruguay, which found that children whose mothers had higher education background had lower risk for stunting.[15] Further study in Bangladesh had emphasized this association, in which study in prenatal to pre-adolescence period had shown strong association between maternal education and stunting in children.[22] The high level of education increases the probability for the mother to provide quality care since prenatal and thus improved child's nutritional intake.[3] Maternal education was a strong determinant factor to children stunting.[15]

However, study results regarding the association of maternal employment status and linear growth are still conflicting. Some previous studies reported that housewife mothers could provide more favorable care for their child than working mothers, by giving more attention to the nutritional intake and maintain environment hygiene. The more time she spends working out of the house, the less time she has to ensure adequate nutritional intake and monitor the child's activity.[23] Working mothers may contribute to the higher family income and may further provide better nutrition for the child and hire caregiver who can take care of the children. On the other hand, working mothers may have less time to supervise their children and prepare their meals.[23] Working mother may have protective impact to childhood stunting if the additional income gained is allocated for better nutrition for children, and mother substitute is available to take care the children.

Regarding paternal socio-demographic factors, there was no significant association between paternal age, education level, and occupation with children linear growth pattern. Fourteen out of 24 subjects who showed declined linear growth had father with more than 9 years of education while other 10 subjects had father with less than 9 years of education. Previous study in low-to-middle-income countries reported similar results which stated that maternal education had bigger impact to children linear growth compared to paternal education.[24] This might be due to the traditional custom in developing countries such as Indonesia and Bangladesh where mother rearing practice and education level as the main caregiver would give stronger impact to the child than father rearing practice and education level. On the other hand, a large population study in Bangladesh and Indonesia showed that paternal education level was associated with children linear growth.[15]

As maternal education is an important risk factor of declined linear growth, ensuring appropriate education for future mothers is a crucial prevention strategy. Parents should encourage their daughters to finish high school before getting married in accordance to our national policy of 12-year compulsory education.[25]

We provided thorough perspective in this study as we looked for the association of children linear growth, not only with maternal socio-demographic factors, but also with the paternal socio-demographic factors. Also, studying as a cohort of term gestational age and normal birth weight children had given better understanding about the association of risk factors and declined linear growth in approximately 18-month span.

However, there were several limitations in this study such as limited number of participants. Our findings were based on bivariate analysis without adjustment for possible confounders. Other demographic and health data such as parental height, family income, family size, spacing between births, nutritional intake might cause confounding bias were not obtained as it was beyond our scope. There was also a 38% drop-out rate in our study even though this drop-out was due to unpredictable natural disaster that resulted in loss of contact. This particular group from flooded area might reflect lower socioeconomic status therefore might cause a selection bias.

In conclusion, mother with education duration less than 9 years was the determining socio-demographic risk factor that contributed to decline linear growth in children age less than 2 years. This finding should be promoted to parents who would encourage their daughters to finish

high school before getting married in order to decrease stunting prevalence, improve cognitive and academic achievement of their children in the future.

Conflicts of Interest

The authors affirm no conflict of interest in this study.

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Community Research

Determinants of HIV provider-initiated testing and counseling screening service used by pregnant women in primary health centers in Surabaya

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ABSTRAK

Latar belakang: *Deteksi HIV secara gratis wajib ditawarkan oleh petugas kesehatan kepada seluruh ibu hamil yang berkunjung ke puskesmas di Surabaya, namun tidak semua ibu hamil memanfaatkannya. Penelitian ini bertujuan untuk mengidentifikasi determinan penggunaan layanan penjaringan HIV melalui* provider-initiated testing and counseling *(PITC) bagi ibu hamil di puskesmas urban di Surabaya. Berbagai penelitian yang telah ada mengenai determinan tersebut kurang komprehensif dan lebih mengutamakan* voluntary counseling and testing *(VCT).*

Metode: *Penelitian ini bersifat potong lintang. Wawancara dilakukan terhadap 150 ibu hamil yang sedang berkunjung ke satu dari 9 Puskesmas di Surabaya dan telah ditawari tes HIV PITC. Kriteria inklusi adalah wanita hamil yang berkunjung ke bagian kesehatan ibu dan anak di Puskesmas. Kriteria eksklusi adalah sudah pernah mendapat konseling HIV sebelum wawancara dan/ atau mengalami gawat darurat obstetrik.* PRECEDE-PROCEED framework*, dipergunakan untuk menggali determinan. dan analisa dilakukan dengan regresi logistik biner dan berganda.*

Hasil: *Penggunaan HIV PITC oleh ibu hamil berhubungan dengan rasa percaya diri untuk diambil darah (p<0,001, adjusted OR=12,368, 95% CI=3,237–47,250) dan penggunaan layanan bidan swasta untuk kehamilan ini sebelum dilakukan tes HIV (p=0,029, adjusted OR=3,902, 95% CI=1,150–13,246). Rasa percaya diri untuk diambil darah untuk tes HIV merupakan mediator efek layanan bidan praktek swasta di masa lalu untuk kehamilan ini terhadap penggunaan layanan penjaringan HIV di Puskesmas.*

Kesimpulan: *Pengalaman penggunaaan layanan bidan swasta meningkatkan rasa percaya diri untuk diambil darah dalam penyaringan HIV PITC, dan rasa percaya diri tersebut meningkatkan penggunaan layanan penyaringan HIV PITC. Diperlukan peningkatan keterlibatan bidan praktek swasta dalam program HIV PITC di Puskesmas.*

ABSTRACT

Background: Offering free HIV screening service for pregnant women in primary health center in Surabaya has become obligatory since 2014, but only 70% used the service. Prior studies on HIV screening mostly focused on Voluntary Counseling and Testing.

Methods: This was a cross-sectional study. Interviews were conducted with 150 pregnant women attending antenatal care in 1 of 9 public health centers (PHCs) in Surabaya and offered HIV screening within the same PHC. The eligibility criterium was pregnant women attending antenatal care in PHCs. The exclusion criteria were having been counseled for HIV prior to the interviews and/or experiencing an obstetric emergency. Using PRECEDE Framework with the concept of a comprehensive framework, this study focuses on identifying determinants of HIV PITC service use in PHCs in Surabaya. Binary logistic regressions and multiple binary logistic regressions were used in analyses.

Results: The service use was associated with self-confidence of getting blood drawn for the test (p<0.001, adjusted OR=12.368, 95% CI=3.237–47.250) and past use of midwife private service for current pregnancy (p=0.029, adjusted OR=3.902, 95% CI=1.150–13.246). Self-confidence of getting blood drawn for HIV test mediated the effect of past use of midwife's private service on HIV screening use.

Conclusion: Past use of midwife's private service affected self-confidence of getting blood drawn for HIV test on HIV screening use, and self-confidence affected the use of HIV PITC. This study results suggest that more midwives' private practices are needed to increase the use of HIV PITC screening in PHC.

Keywords: concentrated HIV epidemic area, HIV PITC screening, pregnant women, urban primary health center

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Human immunodeficiency virus (HIV) screening among pregnant women is essential to detect both the spread of the infection among women and its maternal transmission to children. In 2012, within voluntary counseling and testing context, out of 43,624 pregnant women tested for HIV in 92 hospitals and 13 public health centers (PHCs) in 31 Indonesian provinces providing mother-to-child transmission (MTCT) services, 1,329 (3.01%) were positive.[1] In 2013, the issuance of the Decree of the Minister of Health of the Republic of Indonesia marked the shift of the HIV screening policy from client-initiated testing (CIT) and voluntary HIV counseling and testing (VCT) to provider-initiated testing and counseling (PITC) for pregnant women in low and concentrated epidemic areas in Indonesia.[2] In the new policy, pregnant women are informed about the aim of the screening and consented if they agree to take the procedure. Prior to get their blood drawn, pre-HIV-test counseling is replaced by an informational session while post-HIV-test counseling is obligatory.[3]

Surabaya was appointed as the site of a pilot project of the new policy implementation. In January 2014, an opt-out HIV PITC screening for expectant mothers as part of prevention of MTCT program begin following the World Health Organization (WHO) guidelines.[4] In its implementation, PITC is integrated into the routine antenatal care in every PHC in Surabaya besides the existing hepatitis B and sexually-transmitted infection screenings, urinalysis, nutritional assessment and dental check that take approximately one hour. PITC would affect approximately 47,567 estimated pregnant women in Surabaya, 40% of whom attended antenatal care in PHCs.[5] Private practicing midwives were informed about the new policy and encouraged to refer their clients to PHCs in the areas as they did not meet the qualification to counsel and test. Test positive expectant mothers are referred to appointed public hospitals to start anti-retroviral therapy at no cost. In the mid-year of the pilot project of free HIV PITC screenings in 2014 for expectant mothers, it was noted that not all accepted the screening. Of the 100% targeted pregnant women in PHCs, 30% disagreed to take the test.[6] Therefore, identification of factors contributing to the acceptance of the screening was needed.

PRECEDE framework provides comprehensive and systematic procedures, organized in a conceptual framework of possible relationships. Studies on HIV screening among pregnant women did not either use the comprehensive PRECEDE framework[8–10] or focused on VCT,[11,12] allowing misidentification of which factors that actually played the bigger role. This misidentification could lead to program's financial misallocation of HIV PITC screening. Hence, this study was aimed at identifying determinants of HIV PITC screening service.

This study aimed at determining factors associated with the use of HIV screening service by pregnant women through PITC in Surabaya using diagnostic components of the PRECEDE-PROCEED framework.[7] PRECEDE stands for predisposing, reinforcing and enabling constructs in educational diagnosis and evaluation, while PROCEED stands for policy, regulatory, and Organizational Constructs in Educational and Environmental Development. Only PRECEDE was applied in this study.

METHODS

This was a cross sectional study. Nine PHCs in 9 Surabaya areas where most pregnant women resided were chosen. Convenience sampling was applied for easiness, i.e. for convenience, a day per week was chosen for data collection time. That was the day pre-determined by PHCs as weekly elective antenatal visit day. All eligible pregnant women coming for antenatal care in a chosen day were recruited. New pregnant women came in any day outside the pre-determined day was served by PHCs but were not included in the study. Random sampling could not be applied because the antenatal care (ANC) clinics were walking-in sites. There was no need to make appointments prior to the visits, thus a list of patients where a random sampling would be based on could not be obtained. An interview using a structured questionnaire was conducted by a trained female interviewer in the PHC just after the participant used the routine antenatal care service. Participants were excluded from the study if they have been counseled in other health facilities prior to their visits to the participating PHCs and or were in obstetric emergency situations.

This study used Green's and Kreuter's definitions of predisposing, reinforcing and enabling factors of health service use. Predisposing factors refer to individual characteristics that motivate behavior.

Knowledge, attitude, belief, personal preferences, skills, and self-efficacy fall within this group.[7] In this study, predisposing factors were explored by asking participants to self-report their perception on their knowledge on HIV and answer standard questions on HIV composed by the Joint United Nations Programme on HIV/AIDS UNAIDS[13]: "Can the risk of HIV transmission be reduced by having sex with only one uninfected partner who has no other partners?", "Can a person reduce the risk of getting HIV by using a condom every time they have sex?", "Can a healthy-looking person have HIV?", "Can a person get HIV from mosquito bites?" and "Can a person get HIV by sharing food with someone who is infected?"

Participants were also asked to self-report their self-confidence to get their blood drawn and attend post-HIV-test counseling with this question: "Do you feel that you are confident to get blood drawn/take post-HIV-test counseling?" Participants were also asked to report their awareness of examinations or tests to detect HIV, HIV medication, and prophylaxis for HIV mother-to-child transmission and to rate their self-confidence of using HCT service. These questions were pilot-tested and minor revisions were made. Reinforcing factors refer to "rewards or punishments following or anticipated as a consequence of a behavior."[7] In this study, they were husband's advice to participants to get tested and husband's reminder to participant to get health checked in PHC, assuming that the women anticipated to gain at least verbal rewards after taking their advice. Past use of midwife service for current pregnancy was also a reinforcing factor, assuming that they would get better services when they were referred back to referring midwives. Midwives working in solo private and non-PHC facilities committed to send pregnant women to PHC for HIV checks. The agreement was reached between the Surabayan chapter of the Midwife Professional Association (*Ikatan Bidan Indonesia Surabaya*). After the screening was done, women's were referred back to the referring midwives.

Enabling factors refer to "Environmental characteristics that facilitate action and skills or access to resources or services needed to adopt certain behavior".[7] These factors were explored by asking participants to self-report the order of their current pregnancy, the number of antenatal care they attended in the current PHC they were visiting, the distance between their residences and PHCs, the time needed to access the testing service, their preference on service hours, husbands' advice to get tested for HIV, husbands' reminder to get health checked in the PHC, and whether they used midwife services for the current pregnancy prior to their current visit to PHC. The study flow is presented in Figure 1.

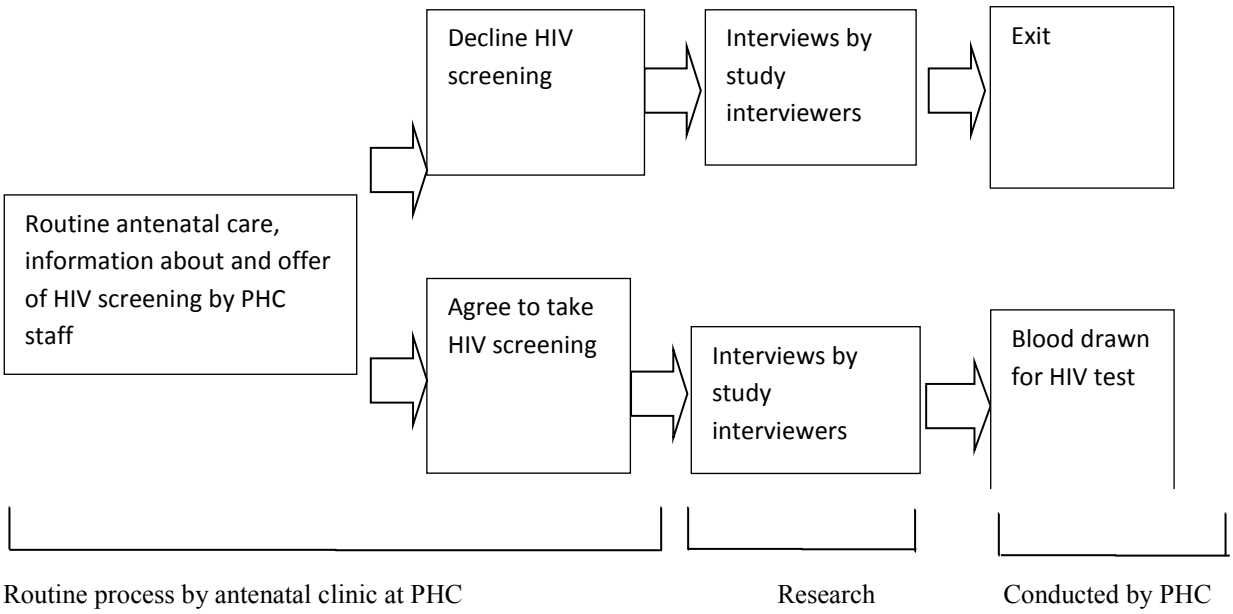

Figure 1. Study flow. PHC= primary health center

Ethical consideration

Ethical permission No. 315/EC/KPEK/FKUA/2014 was granted by the Institutional Review Board of Universitas Airlangga Faculty of Medicine). Participants or their parents were asked for consents before the interviews started and a written informed consent was obtained prior to each interview. Under-aged participants were accompanied by at least one of their parents. Participation in the study was voluntary. All data were kept confidentially. After each interview, participants were told about the correct answers to HIV knowledge questions. Some monetary compensation was provided for the time the participants dedicated to this study.

Data analysis

Categorical data were dichotomously re-coded. "Don't know" and "decline to answer" responses to HIV knowledge questions were treated as incorrect answers and coded "1". "Don't know" and "decline to answer" responses to other questions were treated as missing values. Lower values and orders were coded "1". Multiple imputations[14,15] using Statistical Package for the Social Sciences (SPSS) version 17 was applied to replace missing values. Descriptive analyses were applied to all variables. Determinants of HIV screening service use were analyzed with bivariate binary logistic regressions. Factors with $p<0.05$ were included in the respective multivariate binary logistic regressions. The procedure of Baron and Kenny[16] was run on variables to detect mediation and moderation effect.

RESULTS

Data were collected in 2014 from 150 participants in 9 participating PHCs. Twenty one (14%) participants declined and 129 (86%) agreed to use HIV screening services.

Demographic characteristics

Frequency distribution of demographic characteristics is presented in Table 1.

Predisposing, reinforcing and enabling factors and HIV screening service use

Table 2 presents the detailed frequency distribution of predisposing, reinforcing and enabling factors and the results of bi-variate binary logistic regressions of the use of HIV screening service. Multivariate binary logistic regressions on the PITC HIV use were applied within each group of factors to determine significant associations.

The distance between participants' residence and the service unit ranged from 100 meters to 10 km (mean ± SD=1.8±1.6 km). The time needed to

Table 1. Respondent characteristics and results of logistic regression on the use of HIV screening service

Demographic characteristic	n	%	Crude OR
			Value
Age			
≥28 y.o	75	50	0.491
<28 y.o	75	50	Reference
Ethnic group			
Javanese	122	81.3	1.104
Non-Javanese	38	20.7	Reference
Education level			
Senior High School and universities	99	66	0.810
≤ Junior high school	51	44	Reference
Marital status			
Married, living together	142	94.7	<0.001
Other than married living together	8	5.3	Reference
Hometown			
Surabaya	48	32	1.114
Non-Surabaya	102	68	Reference

None of the respondents' characteristics significantly associated with HIV PITC screening use

Table 2. Frequency distribution of predisposing, reinforcing and enabling factors and results of bivariate and multivariate binary logistic regressions of the use of HIV screening service

Factors	n	%	Crude OR (bivariate)	Adjusted OR (multivariate) within group
Predisposing group				
The order of current pregnancy				
3rd or more pregnancy	36	24	0.320*	0.562
1st or 2nd pregnancy	114	76	Reference	Reference
Perceived self-knowledge of HIV				
Know some	117	78	1.214	
Know nothing	33	22	Reference	
Total score of answers to standard UNAIDS questions				
≥3	10	36.7	0.459	
<3	95	63.3	Reference	
Awareness of examinations or tests to detect HIV				
Aware	138	92	1.588	
Unaware	12	8	Reference	
Awareness of medication to help HIV-infected people live healthy for a longer period of time				
Aware	51	34	1.260	
Unaware	99	66	Reference	
Awareness of prophylaxis for mother-to-child transmission of HIV				
Aware	92	61.3	1.549	
Unaware	58	38.7	Reference	
Self-confidence to get blood drawn for HIV screening				
Confident	138	92	22.795†	12.054†
Not confident	12	8	Reference	Reference
Self-confidence to get post-test counseled				
Confident	116	77.3	4.417†	1.621
Not confident	34	22.7	Reference	Reference
Reinforcing group				
Husband's advice to participants to get tested				
Advised	26	17.3	2.308	
Not advised	124	82.7	Reference	
Husband's reminder to participant to get health checked in PHC				
Yes	125	83.3	2.151	
No	25	16.7	Reference	
Past use of midwife service for current pregnancy (private practice, midwifery clinic)				
Yes	106	70.7	2.824*	3.774*
No	44	29.3	Reference	Reference
Number of antenatal care visit in current PHC				
>2	61	40.7	3.613*	2.975*
≤2	89	59.3	Reference	Reference
Enabling group				
Distance from residence to PHC with HIV screening service				
>1.8 km	40	25.3	2.254	
≤1.8 km	110	71.3	Reference	
Time needed to get to HIV screening service from residence				
>13.7 mins	62	41.3	1.766	
≤13.7 mins	88	58.7	Reference	
Preference on HIV screening service hour				
Current service hour	127	84.7	0.971	
Other service hours	23	15.3	Reference	

*= statistically significant at p<0.05; †=statistically significant at p<0.01

access HIV screening service ranged from 2 to 60 minutes (mean ± SD=13.07±8.4). The number of antenatal care in the PHC ranged from 1 to 9.

A further statistical analysis was applied to predisposing, reinforcing and enabling factors that had been previously proven as statistically significant. Table 3 shows the results. Factors determining the use of HIV screening were self-confidence of getting blood drawn for HIV screening and the past use of midwife's service (Table 3).

The procedure of Baron and Kenny[16] was run, resulting in the mediating effect of self-confidence over the effect of prior use of midwife's service on HIV screening use, meaning that prior use of midwife's service affected self-confidence, then the later affected HIV screening use.

DISCUSSION

The shift in policy from HIV VCT to PITC in Surabaya most likely contribute to involuntary increased acceptance as was found in a systematic review of 10 studies in the US, Scotland and 7 African countries comparing HIV PITC screening and VCT. The non Surabayan studies revealed that the acceptance of HIV VCT versus PITC ranged from 34.8% to 99.9% versus 5.5%–78.7%.[8] The study did not explore aspects that influenced the increased acceptance. In this study, the use of HIV PITC screening service in PHC was higher than it was at the beginning of this study (70% vs 86% among this study participants), indicating that there was an increased adoption of free HIV screening among pregnant women over time. According to the 2015 report, there was also an overall increase to 17,027 acceptances or 76% of the women attending antenatal care offered in PHC or 40% of overall pregnant women in Surabaya.[17] However, the adoption may not fit with stages of change according to diffusion of innovation theory[18], i.e gradual acceptance of an innovation change through persuasion. Diffusion of innovation theory may be more suitable for voluntary counseling and testing (VCT). In line with national policy, the Surabayan HIV PITC is introduced to pregnant women attending antenatal care as a routine examination like the existing hepatitis B, STI screenings and nutritional assessment, in which an informational session is included but the procedure of pre-HIV-test counseling is omitted. Pregnant women who choose to opt-out will be advised to attend VCT sessions where pre-HIV-test counseling is provided regardless the women's decision to take the test. Compared to HIV PITC by pregnant women at its initial phase of study among urban

Table 3. Results of multiple binary logistic regressions of previously shown significant factors on HIV screening service use

Factors	n	%	Adjusted OR	95% CI	
				Lower	Upper
Self-confidence to get blood- drawn for HIV screening					
Confident	138	92	12.368†	3.237	47.250
Not confident	12	8	Reference		
Self-confidence to get post-test counseled					
Confident	116	77.3	3.080	0.945	10.034
Not confident	34	22.7	Reference		
Past use of private midwifery service for current pregnancy (private practice, midwifery clinic)					
Yes	106	70.7	3.528*	1.019	12.212
No	44	29.3	Reference		
Number of antenatal care visit in current PHC					
>2	61	40.7	2.078	0.695	6.213
≤2	89	59.3	Reference		

*= statistically significant at p<0.05 †=statistically significant at p<0.01

Ethiopian (65.5%)[19] in 2007 and Kenyan (84%)[20] in 2008, the Surabayan figure in this study was higher. The difference might due to the different settings, i.e. Surabaya is a low prevalence with concentrated epidemic HIV setting, while in urban Ethiopia the prevalence was 7.7% and rural Kenya (15%). The increasing rates of acceptance of PITC HIV at the end of this Surabayan study was (16% in 10 months) almost similar to the Kenyan's study (14% in 9 months), regardless of the epidemic setting differences. In Cambodia, Myanmar, Nepal, Papua new Guinea, Thailand and Vietnam, pregnant women who knew their HIV status, implying their use of VCT service, were 74%, 35%, 13%, 24%, 94% and 52% respectively.[20] Although those Asian countries have more or less ethnic similarities with Surabayans, several governments of the studied countries might not see that PITC HIV policy was urgently needed as VCT was sufficiently accepted or there was limited manpower resources in case of countries the lower acceptance of HIV testing.

Much has been relied to formal education advancement for remedies to health issues. In this study as well as the Ethiopian study,[19] however, education was not essential in increasing HIV PITC uptakes. Additionally, lack of education on HIV for pregnant women in this study did not associate with HIV PITC uptakes. Further, these findings also confirmed by lack of difference between general knowledge scores of those who refused and agreed to take HIV test. In fact, the policy of HIV screening in antenatal visit does not mention about health workers' obligation prior to HIV testing to elevate women's knowledge about HIV transmission in general, mother-to-child HV transmission and medication to prevent mother-to-child transmission.[2]

More frequent antenatal visits at PHC are also expected to build more pregnant women's trust towards health workers in PHCs, but this was not the case in this study. There was no association between antenatal visit frequency and HIV PITC test uptake found in this study. Interestingly, the test uptake significantly associated with prior antenatal visits to midwives at private practice sites as well as midwifery clinics outside PHCs, general clinics and general hospitals. Midwives' service at these private practices and midwifery clinics usually are small and exclusively designed for maternal services, offering more intimate communication between midwives and patients, and among patients. These midwives are bounded to their professional organization's commitment to send their patients to PHC. Visits to physicians' solo private practices did not relate to test uptakes although it offers more private doctor-patient communication, most likely because the physicians are not interested in sending pregnant women to PHCs for HIV tests, as private physicians know that they are perceived more valuable by the Indonesian community than the more affordable public services at PHCs. In addition, the medical doctor professional organization does not have commitments to send pregnant women to PHCs in support of government's free HIV test policy. These findings are different from the Ethiopian study's[19] where antenatal visit frequency significantly associated with the test uptake in clinics and hospital. The difference might be due to the higher HIV prevalence in Ethiopia that leads to more rigorous testing policy, including patient-health worker communication in the pre-HIV-test counseling.

This study revealed that test uptakes significantly related to self-confidence to get blood drawn for HIV test. With an adjusted OR of 12.368, self-confidence to get blood drawn was the most important factor for the test uptake. Again, trust might have a role in that self-confidence development gained from more intimate communications between midwives and patients in for women only facilities. From the trust gained, midwife might encourage patients to take an HIV test that cannot be more painful than to get blood drawn like in other existing antenatal laboratory tests. While mother-to-child HIV transmission may be halted through increased HIV PITC testing and medication, incomplete-consented patients for this testing can be an ethical issue.

C Framework that was applied in this study considered both husband's and midwife's roles. However, this study did not find the role of husbands on the women's decision to take HIV screening. In Indonesia, detailed domestic health businesses within the households have traditionally belonged to female's domain. This is different from results of other studies conducted in other areas of similar epidemic level[9,10] that underlined the husbands' or male partners' role of discouraging women's decision to take HIV screening. In those studies, worry about husbands' disapproval to take HIV test

was the reason of declining the test, implying that the husbands doubted the institutions credibility or already knew about the consequence of being tested positive. In Kenya, where HIV prevalence is higher than Surabaya, anticipated stigma from male partners was strongly associated with HIV test refusal.[11]

In line with our study findings, husbands may be targeted by the PHC to increase their wives' antenatal HIV screening at PHCs as husbands' reminder is important to the wives to get to the PHCs. Efforts to generate more knowledgeable husbands needs to be carefully designed in order to support pregnant women's decision in such ways that will not result in women's discouragement on using HIV screening service.

The implementation of HIV screening policy could be improved if midwife service is expanded to HIV education for women so that their self-confidence to take HIV test will also be developed based on increased knowledge in addition to trust or other non-rational processes. Inside the PHCs, an already missed opportunity of educating pregnant women about HIV before HIV test should be recaptured by providing an information session or distributing printed materials.

The contrasting results of this study compared to previous studies' suggest that midwives in urban, concentrated HIV epidemic region of Surabaya can be better prepared to boost pregnant women's self-confidence in order to increase the HIV screening uptake. The service of midwives in their private practice has not been covered by the national health insurance scheme through direct financing, but rather through their networking with primary care institutions. This issue should be addressed by including midwives' service to improve access that may lead to increased HIV PITC use.

The extrapolation of the study results may be limited to urban primary health centers where midwives' services are available in solo private practices, primary health cares and midwifery clinics. The external validity of the study may be improved through random sampling. In addition, the study was cross-sectional in nature. Hence the directions of associations remain vague. A bigger sample size may be needed to avoid under-power. As only 40% pregnant women used antenatal services in PHC, studies on HIV PITC screening in other primary health care facilities are needed. Physician's role in advising pregnant women to get HIV checks at PHC needs to be studied.

In conclusion, HIV PITC screening use was affected by a predisposing factor, i.e self-confidence of getting blood drawn for HIV test. The self-confidence was affected by past use of midwife's service affected on HIV screening use. The use of HIV screening service at PHCs may be indirectly improved by increasing husbands' involvement in reminding their wives to get their health checked.

Conflict of interest

The authors affirm no conflict of interest in this study.

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Case Report

Pediatric gunshot penetrating head injury: a case report with 2-year follow-up

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ABSTRAK

Kejadian cedera kepala akibat luka tembak senjata api sangat jarang dijumpai dan umumnya korban meninggal sebelum dibawa ke unit gawat darurat (UGD) rumah sakit. Studi kasus ini melaporkan seorang anak laki-laki berusia 12 tahun yang ditembak oleh seorang temannya menggunakan senjata revolver *dengan maksud bercanda. Peluru masuk dari mid frontal dan keluar dari oksipital kanan. Pada pemeriksaan dijumpai tanda vital stabil dengan GCS 8. Penanganan yang rasional memberikan hasil yang baik. Komplikasi yang dijumpai berupa hidrosefalus dan memerlukan pemasangan* VP-shunt*. Cacat pada tulang kepala ditutup dengan menggunakan mesh titanium. Pemantauan selama 2 tahun menunjukkan hasil yang baik. Pasien mampu melakukan aktivitas secara mandiri dan bersekolah kembali.*

ABSTRACT

Gunshot is a rare subset of penetrating head injury, and generally the victim dies before arriving at the hospital. This paper reported a case of an intracranial gunshot injury in a 12 year-old boy that was shot by his friend, whose primary intention was to play around, using a revolver. A missile projectile penetrated from mid frontal and came out from right occipital. Vital signs were stable with GCS 8 from physical examination. A rational management strategy should permit a good outcome. The only complications that occured were hydrocephalus, yet it was managed by VP–shunt. Skull defect was closed using titanium mesh. A two-year follow-up showed a good result. The patient was able to do daily activity and back to school again.

Keywords: gunshot penetrating head injury, pediatric

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The management of gunshot penetrating head injury is challenging for neurosurgeon, especially in pediatrics due to scarcity of experience and literature. Using clinical corollary literature on adults to pediatrics should be carefully considered.[1] Gunshot penetrating head injury is usually associated with crime and suicide attempts with mortality rate 70–90% die before hospitalization and 50% die in hospital during resuscitation. According to several literatures, pediatrics has better outcomes in overall mortality and greater tendency for neurological recovery than in adults.[1,2]

Management of penetrating brain injury focusing solely on head injury should be avoided. Instead, a thorough review by primary and secondary survey of advance trauma life support (ATLS) is highly recommended. Radiology should be performed after the patient is stable. Computer tomography-scan (CT-scan), Skull X-ray anteroposterior (AP) and lateral are the most common and useful tools to evaluate gunshot head injury. The addition of CT 3D, CT angiography, and digital subtraction angiography (DSA) provide a reasonable initial assessment when available. The gunshot wounds can be treated using medication and by surgery. The purpose of medication is to decrease intracranial pressure (ICP) by preventing brain edema (head elevation 30–45°, hyperventilation $PaCO_2$= 30–35 mmHg, mannitol), antiepileptic drugs, antibiotics administration, stress ulcer prevention, and administration tetanus toxoid. Moreover, surgery attempts to prevent not only a secondary injury caused by increasing ICP, but also infection and ischemic. Usual surgery procedures include brain stem decompression, hemostatis, and evacuation of mass lesion like hematoma, bone fragment, missile residual, and the repair of wounds.[3,4]

CASE REPORT

After getting permission from the patient's parent, we reported a case of 12-year-old boy who was shot at the head by his friend, whose primary intention was to play around, using his father revolver. The patient arrived at Haji Adam Malik Hospital 10 hours after the incident with chief complaint decrease of consciousness without any history of vomiting and seizure. According to physical examination, vital signs were stable with glasgow coma scale (GCS) E2M4V2, pupils are bilaterally equal and reactive, and no lateralization was documented. Based on head inspection, missile was found entering from mid frontal and coming out from right occipital. No other extra-cranial injury was found.

Laboratory blood investigations showed leukocytosis 30,000/mm^3, increasing international normalized ratio (INR)1.34, D-dimer 1,600 ng/mL, and hyponatremia 132 mEq/L. Head CT scan showed fracture on the frontal and right occipital,intracerebral hematoma along with the missile track from frontal through right occipital, extensive perifocal edema, basal cistern compression, and midline shift more than 5 mm.

Craniectomy and intracerebral hematoma evacuation with source bleeding control wereperformed to the patient. A right pterional incision was done for frontal approach,meanwhile a horse shoe incision was conducted for exit wound approach. Dura was found torn with brain prolapse, source bleeding from cortical artery at the entry wound, and sigmoid sinus at the exit wound. Operation field was then washed with normal saline and hydrogen peroxide several times. Hematoma evacuation and bleeding control

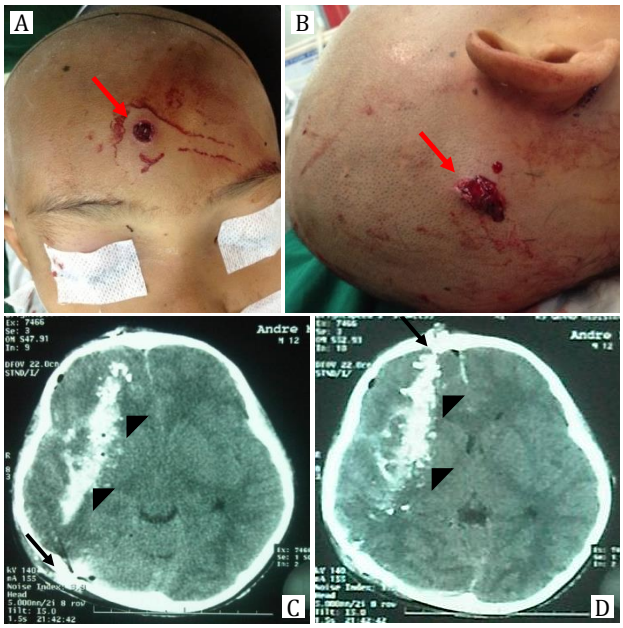

Figure 1. A,B) Missile enters from mid frontal and comes out from right occipital (red arrow); C,D) Head CT scan shows fracture on the frontal and right occipital, intracerebral hematoma (black arrow) along the missile track from frontal through right occipital (arrowhead). Sulcus and gyrus are hard to visualize. Extensive perifocal edema and midline shift are more than 5 mm

with bipolar and surgicel® were also conducted. As missile residual was not found, we continued with duraplasty using fascia. The wound was washed again with saline and hydrogen Peroxide. Finally, the bone was removed, and the skin was closed.

The patient was administered in pediatric intensive care unit (PICU) for 7 days and used the ventilator for 5 days. During the treatment at PICU, the patient was given antibiotic (Ceftriaxone 1 gr/12 hours/iv and metronidazole 500 mg/8 hours/iv), seizure prophylactic, and mannitol. A CT-scan control on the second day after operation showed absorption of hematoma with perifocal edema, midline shift less than 5 mm, and uncompressed basal cistern. Seizure prophylactic was administered for 7 days and was stopped without any episodes of seizure. Patient was discharged after 15-day care at hospital with GCS 15 and left-sided hemiparesis with muscle strength was 4. Patient was followed up regularly at neurosurgical clinic and had regular physiotherapy.

Two months after the discharge, defect was bulging and tense without any episode of vomit and seizure. After regular physiotherapy, left-sided hemiparesis was recovered, and muscle strength was back to 5. Head CT scan was performed with a result of skull defect on the right front temporal and right occipital, cerebromalacia along the missile track and filled with cerebrospinal fluid, dilatation of right ventricle lateral, open basal cistern, and no midline shift. Ventriculoperitoneal (VP) shunt was placed on the left keen. Bulging was reduced after VP shunt, and defect became sunken. Then cranioplasty was performed using titanium mesh to close the skull defect. Patient was discharged in a good condition after 5-day treatment. Patient was still followed up regularly until two years after his first operation at neurosurgical clinic with a good result of neurologic function. Patient can do daily activity independently and was back to school.

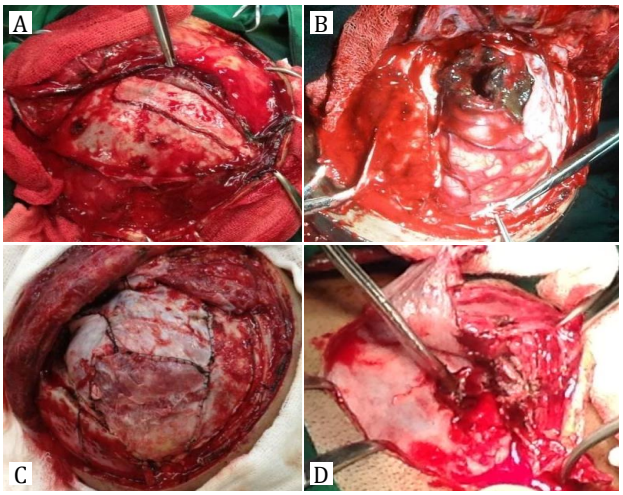

Figure 2. During the operation, A) Missile entered from frontal and caused linear fracture, thus debridement was conducted; B) After opening the dura, brain was prolapsed with subarachnoid hemorrhage and bulging; C) After hematoma evacuation, duraplasty with fascia was performed; D) Exit missile caused skull fracture and brain prolapse

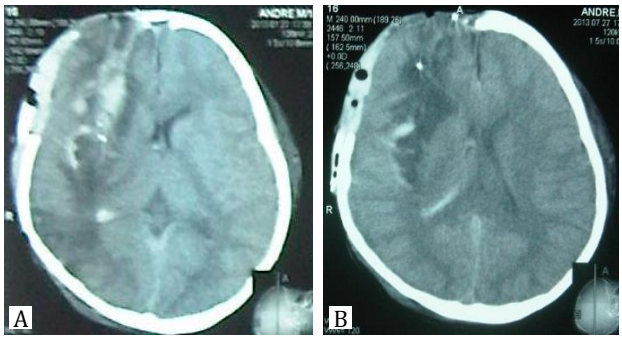

Figure 3. Head CT-scan control after 2-day operation shows absorption of hematoma with perifocal edema and midline shift less than 5 mm

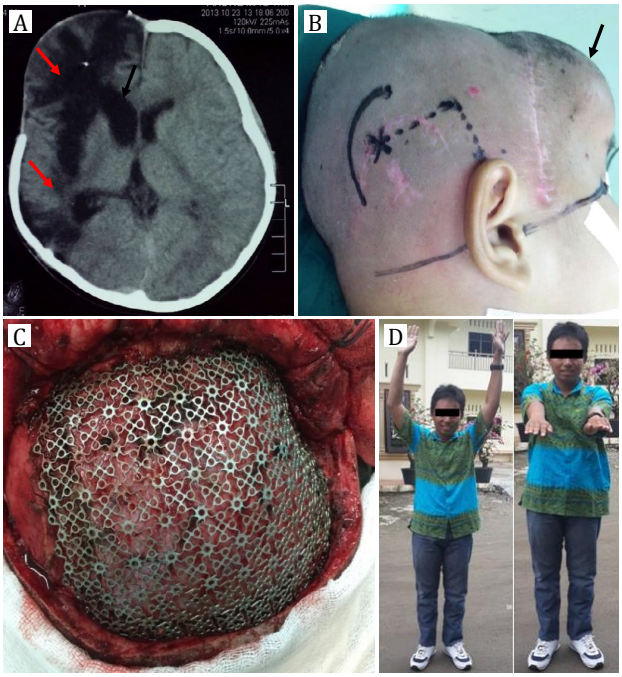

Figure 4. A) Two-month head CT scan after first operation, cerebromalasia along missile track fills with CSF (red arrow) and dilatation of right ventricle lateral (black arrow); B) Design incision and puncture point for VP shunt also shows bulging on the right frontal (black arrow); C). Cranioplasty with titanium mesh after VP shunt; D) Clinical presentation after 2-year follow-up shows a good outcome without any neurologic deficit

DISCUSSION

A gunshot wound to the head is a rare case in emergency rooms, especially in pediatrics. Gunshot penetrating head injury has a high mortality rate around 90% in adults and lower in pediatrics, around 65%. The highest mortality rate is between 70–90% before hospitalization and 50% of the survivals die during resuscitation in emergency rooms. Therefore, the management of gunshot penetrating head injury is very challenging for neurosurgeon, especially in pediatrics due to the scarcity of experience and literature. We could not find any reports of pediatric gunshot penetrating head injury in Indonesia. The use of clinical corollary literature and experiences on adults to pediatrics should be carefully considered.[1,5]

As a general for traumatic brain injury, pediatrics has a better propensity for neurological recovery than adult population. This caused difficulties in deciding appropriate management and predicting the prognostic. The only tool to guide management decisions and to provide prognostic information for gunshot to the head in pediatrics is St. Louis scale. Positive predictive value of ±90% for survival with St. Louis score ≤4, and negative predictive value of 78%–96.7% for death with St. Louis score ≥5. In our case, patient had score 3 with 2 points from 3 lobes involvement and 1 point from midline shift. Our limitation is that we could not perform ICP monitoring due to the lack of facility. We assumed that ICP was controlled post-operatively from good responses in vital sign and neurologic examination during care at pediatric intensive care unit. In literature, ICP monitoring was also routinely performed post-operatively than pre-operatively.[1,6]

Decompressive craniectomy around the missile entrance site has been a favored procedure in previous military conflicts. The present recommendation is to perform craniotomy and debridement of the skull with replacement of the bone to avoid cranioplasty in the future if the brain is not severe swelling. Decompressive craniectomy where the bone flap stored in a tissue bank or abdominal wall for future reimplantation was not recommended because of the increased risk for contamination and infection at the implanted site. In this case, decompressive craniectomy was performed because severe brain edema and bone flap were removed. Operation field washed using normal saline and hydrogen peroxide to reduce risk of infection. Then cranioplasty using titanium mesh was conducted to close the skull defect in the next operation.[7,8]

Decompressive craniectomy will make physiological change in intracranial pressure, cerebrospinal fluid circulation, and cerebral blood flow that caused many complications. A common complication after traumatic brain injury is hydrocephalus with reported incidence between 0–88%. Hydrocephalus will usually occur 1-month post-surgery. Pathophysiology hydrocephalus was caused by CSF malabsorption or obstruction of cerebrospinal fluid (CSF) flow and diminished CSF pulse wave caused by transmission of pulse out through the skull defect. Management for this complication is using VP shunt procedure. In this case, hydrocephalus occured on the second month after surgery. The area of cerebomalasia filled with CSF caused by connection to the right lateral ventricle. Increasing of CSF volume caused herniation and bulging through skull defect. This complication could be managed well by VP shunt procedure with diminished of the bulging after the procedure.[9,10]

One of the significant factors in the recovery from traumatic brain injury (TBI) is age. Older adults showed less favorable outcomes than younger adults. De la Plata et al[11] analyzed the effects of TBI to the brain function using disability rating scale (DRS). The research showed that the 16–26 year old age group has the greatest improvement in disability and lower decline than older patients. It was caused by the increased plasticity of the brain in younger people. Subsequent research showed opposite pattern, young children with immature brain also showed less capable of recovering from damage. Anderson et al[12] learned about the effects of TBI in developing brain by analyzing cognitive outcomes. The research showed that older children (8–12 years) had better cognitive recovery patterns than the younger children (3–7 years).[11–13]

Conclusion, pediatric gunshot penetrating head injury is scarce and challenging case. This patient has good outcome consistent with St. Louis scale for pediatric gunshot head injury. Positive predictive value of ±90% for survival

with St. Louis score ≤4 and our patient had St Louis score 3.

Conflict of interest

The authors affirm no conflict of interest in this study.

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