HDAC2 and PCNA expression is correlated to decreasing of endoxifen sensitivity in human breast cancer stem cells ALDH+

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Abstrak

Latar belakang: *Sel punca kanker payudara (breast cancer stem cells/BCSC) adalah subpopulasi sel kanker yang memiliki kemampuan menghasilkan tumor baru dan bersifat seperti sel punca. Penelitian kami sebelumnya menggunakan jaringan kanker payudara mengungkapkan bahwa ekspresi gen* histone deacetylase 2 *(HDAC2) dan* proliferating cell nuclear antigen *(PCNA) ditemukan perbedaan signifikan setelah terapi neoajuvan hormon dan kemoterapi. Penelitian ini bertujuan untuk menganalisis hubungan antara ekspresi HDAC2 dan PCNA dengan kelangsungan hidup sel punca kanker payudara dengan penanda* aldehyde dehydrogenase + *(ALDH+) yang diberi perlakuan endoksifen.*

Metode: *Sampel adalah BCSC primer manusia ALDH+ yang diberi perlakuan endoksifen 4 uM masing-masing selama 2, 4, 6, 8, 10, 12, 14 hari. Viabilitas sel dilihat dengan menggunakan trypan blue dan ekspresi mRNA HDAC2 dan PCNA ditentukan menggunakan qRT-PCR.*

Hasil: *Viabilitas BCSCs ALDH + menurun setelah 2 sampai 4 hari pemberian endoksifen. Pada periode ini juga didapatkan ekspresi mRNA HDAC2 dan PCNA mengalami penurunan. Tetapi setelah pemberian endoksifen selama 8 hari, viabilitas BCSCs ALDH + mengalami peningkatan dan ditemukan peningkatan yang signifikan pada hari ke-14 pemberian endoksifen. Ekspresi mRNA HDAC2 dan PCNA juga menunjukkan peningkatan mulai pada hari ke-8 dan terus meningkat hingga hari ke-14 pemberian endoksifen. Penelitian ini menunjukkan pola yang sama antara ekspresi mRNA HDAC2 dan PCNA dan viabilitas sel.*

Kesimpulan: *Induksi endoksifen yang lama menurunkan sensitivitas efek endoksifen pada BCSC manusia dan ekspresi HDAC2 dan PCNA berkorelasi dengan viabilitas BCSC manusia setelah induksi endoksifen.* ***(Health Science Journal of Indonesia 2019;10(2):77-81)***

Kata kunci*: sel punca kanker payudara, viabilitas sel, HDAC2, PCNA, endoksifen*

Abstract

Background: Breast cancer stem cells (BCSCs) are subpopulation of cancer cells that has the ability to generate new tumor and similar properties to stem cell. Our previous study using breast cancer patients revealed that gene expression of histone deacetylase 2 (HDAC2) and proliferating cell nuclear antigen (PCNA) were significantly altered after neoadjuvant hormone and chemotherapy. This study aimed to analyze the correlation between HDAC2 and PCNA expressions with the viability of breast cancer stem cells aldehyde dehydrogenase + (BCSC ALDH+) treated by endoxifen.

Method: Samples are human primary BCSCs ALDH+ that treated with 4 uM of endoxifen for 2, 4, 6, 8, 10, 12, 14 days, respectively. Cell viability was measured using trypan blue exclusion assay and the mRNA expressions of HDAC2 and PCNA were determined using qRT-PCR.

Results: The viability of BCSCs ALDH+ was decreased after 2 days until 4 days-endoxifen treatment. It also demonstrated that mRNA expression of HDAC2 and PCNA were decreased in this period. But after 8 days-endoxifen treatment, the viability of BCSCs ALDH+ was increased. The increasing of viability was higher in 14 days-endoxifen treatment. The mRNA expression of HDAC2 and PCNA also showed increasing begin on 8 days and continued to increase until 14-days endoxifen treatment. We found a similar pattern between HDAC2 and PCNA expression and cell viability

Conclusion: Prolonge endoxifen treatment decrease sensitivity of endoxifen effect in human BCSC and the expression of HDAC2 and PCNA are correlated to human BCSCs viability after endoxifen treatment. ***(Health Science Journal of Indonesia 2019;10(2):77-81)***

Keywords: human breast cancer stem cells, viability, HDAC2, PCNA, endoxifen

Epigenetic modifications play an important role in regulating biological processes. Histone deacetylation is one of epigenetic modifications that regulated by an enzyme family of histone deacetylases (HDACs). This enzyme will remove acetyl group from histones and resulting in a non-permissive chromatin conformation that suppress some genes transcription activities. Cancer is one of the diseases that could be affected by epigenetic alteration. Some studies reported that an increase in histone deacetylation causes increased cell proliferation, cell migration, angiogenesis and invasion by reducing transcription of tumor suppressor genes.[1,2]

Proliferating cell nuclear antigen (PCNA) is known as a molecular marker for proliferation because of its role in DNA replication. This protein is found overexpressed in cancer cells that have high proliferation.[3] PCNA has proven correlated to worse disease progression and prognosis in cancer.

Approximately 70-80% of breast cancer has overexpression of estrogen receptor (ER). Breast cancer with hormone receptor (ER) positive has a good response to anti-estrogen or aromatase inhibitor as first line drug. Endoxifen is one of hydroxylated tamoxifen metabolit (4-hydroxy-N-desmethyl-tamoxifen) and significantly more potent than tamoxifen in its ability to bind to ER, and in suppression of ER-dependent breast cancer proliferation.[5]

Breast cancer is a heterogenous disease which consists of various type of cells including cancer stem cells. Breast cancer stem cells are known as a minor population among breast cancer cells that have capability to self renew, promote tumor growth and differentiate into all cell types in a tumor.[6] Our recent study investigated gene expression profiles of stem cell and p53 in advanced breast cancer using next generation sequencing has reported that expression HDAC2 and PCNA is significantly altered after neoadjuvant hormone and chemotherapy.[7,8] This study aims to investigate the mRNA expression of HDAC2 and PCNA in human BCSC ALDH+ during endoxifen treatment and its correlation to cell viability.

METHODS

Samples are primary culture of human BCSCs ALDH+ which obtained from previous study that has isolated the cancer stem cells by ALDH1 marker (flowcytometer). The cells were grown in serum free medium DMEM F12 with 1% penicillin/streptomycin and 1% amphotericin B and incubated in 5% CO2 at 37˚C.

Cytotoxic Assay

Approximately 10^3 cells/well (96-microwell-plate) of human BCSCs ALDH+ were incubated in DMEM F12 with 5% CO2 at 37˚C and after 24 hours, the medium was replaced with 100 µL of fresh medium for control and fresh medium containing various concentration of endoxifen (0.1, 0.5, 1, 5, 10 and 20 µM) for treatment group. Endoxifen-treated cells were incubated in 24 hours and the relative viable cells number was determined by MTS assay method (Promega®). After 24 hours, the medium culture was replaced with 120 µL fresh medium containing MTS:PMS ratio=20:1 and incubated in 1-4 hours. After the brown colour appears, the absorbance was read at 490 nm using microplate reader (Varioskan®). The 50% cytotoxic concentration (CC50) was determined from the dose-response curve. We determined the endoxifen concentration for the cells treatment not higher than CC50 concentration.

Endoxifen treatment

Approximately 10^5 human BCSCs ALDH+ were plated each well in 12 well-plate and incubated overnight. Medium was replaced by endoxifen-treated medium 4 µM (according to cytotoxic assay result) for treatment group and only complete medium DMEM F12 for the control group. Treatment was conducted during 2nd, 4th, 6 th, 8th, 10th, 12 th and 14th day and every 2 days the medium was refreshed and the cell number was counted both in endoxifen-treated and control group to determine the cell viability using trypan blue exclusion method. We collected the cells and isolated the total RNA using Tripure isolation reagent kit (Roche®) according to the manufacturer's instructions.

Quantitative RT-PCR

The concentration of RNA was measured using spectrofluorometer (Varioskan, Thermo Scientific). One-step RT-PCR was carried out using ECO48® real time qPCR system (PCRmax). The RT PCR was performed in 20 µL volume with a 45˚C incubation for 5 minutes initially, followed by a 3-minute incubation at 95˚C, then 40 cycles of 95˚C for 5 seconds, 60˚C for 30 seconds, 72˚C for 30 seconds, and melting curve incubation. We used 18sRNA as housekeeping gene. The primer sequences for quantitative RT-PCR are shown in table 1. The relative expression is produced by comparing Ct value of treatment group to control group and it is calculated by Livak formula.[9]

Table 1. Primer sequences for HDAC2, PCNA and 18sRNA

No.	Gene	Primer Sequence	Product
1.	HDAC2	F: 5'- CCA TAA AGC CAC TGC CGA AG -3' R: 5'- CAC AGC TCC AGC AAC TGA AC -3'	199 bp
2.	PCNA	F: 5'- CTT CCC TTA CGC AAG TCT CAG -3' R: 5'- TTG AGT GCC TCC AAC ACC TT -3'	189 bp
3.	18sRNA	F: 5'- AAA CGG CTA CCA CAT CCA AG -3' R: 5'- CCT CCA ATG GAT CCT CGT TA -3'	155 bp

Ethical Declaration

This study has been approved by the Health Research Ethics Committee Faculty of Medicine Universitas Indonesia – Cipto Mangunkusumo Hospital number 390/H2.F1/ETIK/2014.

RESULTS

Cytotoxic Assay Analysis

The cytotoxicity of endoxifen on primary human breast cancer stem cells (ALDH+) was determined by calculation of CC50 using several endoxifen concentrations (0.01, 0.05, 0.1, 0.5, 1, 5, 10 and 20 µM). The curve of CC50 for endoxifen was created based on endoxifen concentration againts the absorbance in log10 (figure 1). We got the CC50 of endoxifen on human breast cancer stem cells ALDH+ is 6 µM. Based on the cytotoxic assay result and optimization trial, we determined the endoxifen concentration is 4 µM for BCSC induction.

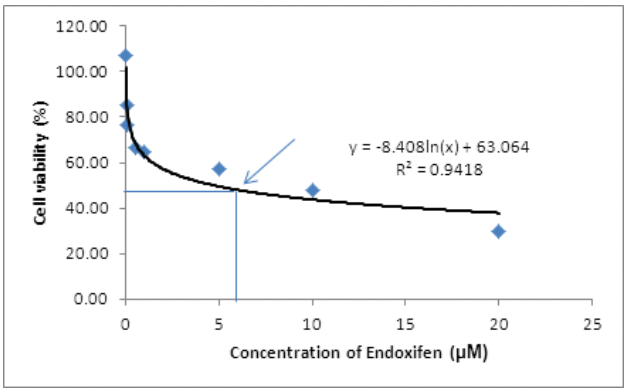

Figure 1. Cytotoxic concentration 50 of endoxifen treatment in human BCSC ALDH+. It shows the CC50 of endoxifen is 6 µM.

Cell Viability

The number of cell viability was shown as the ratio (%) of live cell number in endoxifen group devided by live cell number in control group. Cell viability in endoxifen group appears to decrease in 2nd-day (51%) and persistant until 6th-day (58%), but the cell viability found significantly increased after 8th-day (82%) and more increased until 14th-day (99%) compare to control group (figure 2).

The mRNA expression of HDAC2 after endoxifen treatment

HDAC2 expression was decreased after 2 day-treatment of endoxifen (0.790) compare to control group and it still decreased in 6 day-treatment (0.502). The expression was significantly increased in 10 and 14 day-treatment (1.429 and 2.633) compare to 2 day-treatment groups (figure 3).

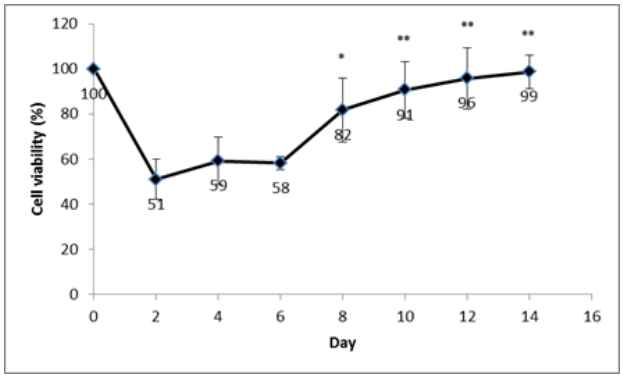

*Figure 2. Cell viability of human BCSC ALDH+ treated by endoxifen 4 µM. Cell viability is rasio (%) of live cells in endoxifen treatment group compare to live cells in control group during 14 days treatment. Significancy compared to 2-day group (*p<0.05 and **p<0.001).*

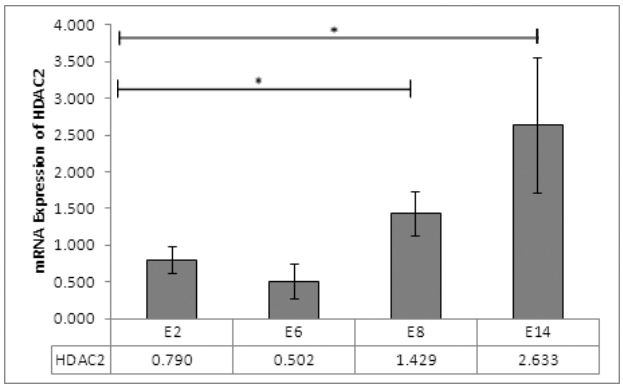

*Figure 3. The mRNA relative expression of HDAC2 in human BCSC ALDH+ during 14 days treatment of endoxifen 4 µM. Significancy compared to 2-day treatment (E2) group (*p<0.05).*

The mRNA expression of PCNA after endoxifen treatment

PCNA expression also decreased after 2 day-treatment of endoxifen (0.767) compare to control group and it still decreased in 6 day-treatment (0.447). The expression was significantly increased in 10 and 14 day-treatment (1.777 and 3.843) compare to 2 day-treatment groups (figure 4).

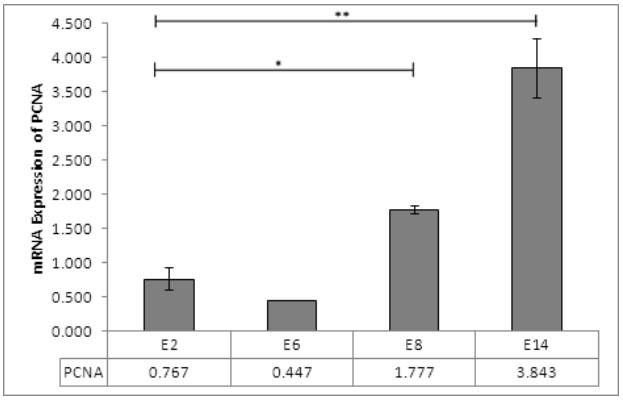

*Figure 4. The mRNA relative expression of PCNA in human BCSC ALDH+ during 14 days treatment of endoxifen 4 μM. Significancy compared to 2-day treatment (E2) group (*p<0.05 and **p<0.001).*

DISCUSSION

According to the cytotoxic assay result, we got the CC50 of endoxifen on human breast cancer stem cells ALDH+ is 6 μM. We also performed the optimization trial with several concentrations that lower than CC50. We determined the endoxifen concentration to BCSC treatment is 4 μM, because among the below CC50 concentration, this concentration gave the best inhibition towards BCSC proliferation.

In this study, we demonstrated that the cell viability of human BCSCs was decreased after endoxifen treatment in the early period (2-day until 6-day treatment) however it would increase in late period (8-day until 14-day treatment). The mRNA expression of HDAC2 and PCNA also have the same pattern as cell viability after endoxifen treatment. It was decreased in the early period (2-day until 6-day treatment), but the expression would increase after 8-day until 14-day treatment. It showed us that the expression of HDAC2 and PCNA contributed to the human BCSCs cell viability.

Breast cancers are comprised of a highly heterogeneous population of cells, including the small population possess the ability to regenerate tumors in vivo. Another study was proved that histone deacetylases (HDACs) play essential roles in the cancer stem cells phenotype.[10] Histon deacetylation is one of genetic modification that regulated by HDACs. Histone deacetylases (HDACs) regulate the expression and activity of numerous proteins involved in cancer initiation and cancer progression. By removal of acetyl groups from histones, HDACs create a non-permissive chromatin conformation that prevents the transcription of genes that encode proteins involved in tumorigenesis. In addition to histones, HDACs also deacetylate a variety of other protein targets including transcription factors and other abundant cellular proteins involved in control of cell growth, differentiation and apoptosis.[11] There are eighteen isoenzymes of HDACs that have already known but only class 1 HDAC (HDAC 1, 2, 3 and 8) that reported involved in cancer.[12]

Endoxifen was proved to have better antiestrogen capacity compare to tamoxifen through ERα degradation and blocking ER activity.[5] Some evidence suggests that ERα signaling has the potential to contribute to epigenetic alteration. Estrogen stimulation is shown to induce several histone modifications at the ERα target gene promoters such as acetylation, phosphorylation and methylation through interaction with histone modifying enzymes.[13] One mechanism of drug resistance is overexpression of efflux transporters such as ATP binding casette subfamily B member 1/ABCB1 (P-glycoprotein), where its expression is also found high in breast cancer stem cells.[14]

Proliferating cell nuclear antigen (PCNA) is a cofactor of DNA polymerases that coordinate several functions in the replication process. Some studies reported that HDAC1 interacts with PCNA and HDAC1 and 2 are associated with newly replicated DNA.[15] Another study demonstrated that HDAC inhibitors inhibit cell cycle progression and kill cancer cells by triggering DNA damage during DNA replication.[16]

The increasing of cell viability after endoxifen treatment showed that the sensitivity of this treatment was decreased. It is showed by the viability in 8-day treatment was begin increased (figure 2). The decreasing of sensitivity treatment is early mechanism of drug resistance. In this study has revealed the high expression of HDAC2 and PCNA is associated with decreasing of endoxifen effect. It showed by the expression of HDAC2 and PCNA were begin increased in 8-day until 14-day treatment

(figure 3 and 4). Other study reported that HDAC2 overexpression correlated with the metastasis, progression and the increased Ki67, multidrug resistance protein expression in breast cancer.[17] HDAC2 will remove acetyl groups from histones and creates a closed chromatin structure that prevents the transcription of genes involved in growth arrest, differentiation, and apoptosis.[11] The expression of PCNA is correlated with high cytological grading and poor prognosis in renal carcinoma.[18] PCNA enhances the processivity of DNA polymerase ε which conducted DNA replication. Beside DNA synthesis, DNA polymerase ε is involved in DNA damage revision, so it will increase the ability of cancer cells to avoid apoptosis.[19]

In conclusion, prolonged endoxifen therapy can cause decreasing in endoxifen effect which can lead to resistance. The increasing of HDAC2 and PCNA expressions correlated to the decreasing of endoxifen effect in human BCSC that showed by the increase of cell viability in late group of endoxifen treatment.

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Cloning and expression of Human Papilloma virus type 16 L1 capsid protein in bacteria

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Abstrak

Latar belakang: *Secara alamiah protein kapsid L1 Human Papillomavirus (HPV) tipe 16 dapat mengalami auto assembly untuk membentuk Viral like particle (VLP). Terkait dengan penelitian vaksin HPV, VLP dapat digunakan untuk berbagai keperluan seperti vaksin, pseudovirion atau SpyTag-Spycatcher. Penelitian ini ditujukan untuk mendapatkan plasmid rekombinan yang digunakan untuk produksi protein L1 HPV 16.*

Metode: *Gen penyandi protein L1 HPV 16 diklona ke dalam vector pQE80L, suatu plasmid yang mengandung sistem ekspresi untuk prokariota. DNA penyandi HPV 16 L1 disisipkan pada situs restriksi BamHI dan Hind III plasmid pQE80L. Plasmid rekombinan yang mengandung gen L1 HPV 16dikonfirmasi menggunakan PCR dan analisis enzim restriksi. Lebih lanjut untuk memastikan bahwa gen rekombinan L1 HPV 16 dapat diekspresikan dalam prokariota, plasmid rekombinan ditransformasikan ke bakteri Escherichia coli BL21 (DE3). Bakteri diinduksi dengan Isopropyl β-D-1-thiogalactopyranoside (IPTG) dengan berbagai konsentrasi dan berbagai waktu inkubasi.*

Hasil: *protein rekombinan L1, berat 56 kDa, telah berhasil diekspresikan dalam sistem prokariota. Protein rekombinan L1 dapat dimurnikan menggunakan Talon^R dalam kondisi denaturasi.*

Kesimpulan: *gen L1 HPV 16 telah dikloning ke dalam pQE80L dan berhasil diekspresikan dalam sistem prokariota.* ***(Health Science Journal of Indonesia 2019;10(2):82-9)***

Kata kunci: *L1, HPV 16, cervical cancer*

Abstract

Background: Naturally Human Papillomavirus (HPV) type 16 L1 capsid protein can auto assemble to form Viral like particles (VLP). Concerning to vaccine development for HPV, VLP can be used for a variety of needs such as a vaccine, pseudovirion or SpyTag-Spycatcher. In this study, to obtain a vector expression that can be used in the production of HPV L1 protein, we cloned gene coding HPV 16 L1 protein into pQE80L a plasmid contains an expression system for prokaryote.

Methods: The DNA coding HPV 16 L1 was inserted at BamHI and Hind III restriction sites of pQE80L plasmid. The recombinant plasmid containing the HPV L1 gene was confirmed using PCR colony and enzyme restriction. Further to ensure the recombinant HPV 16 L1 gene could be expressed in a prokaryote, the recombinant plasmid was transformed into bacteria Escherichia coli BL21 (DE3). The bacteria were induced with IPTG with various concentrations and various incubation time.

Result: L1 recombinant protein, 56 kDa in weight, has successfully been expressed in prokaryote system. L1 recombinant protein can be purified using Talon^R under denaturing conditions.

Conclusion: L1 HPV 16 gene has been cloned into pQE80L and successfully expressed in prokaryote system. ***(Health Science Journal of Indonesia 2019;10(2):82-9)***

Keywords: L1, HPV 16, cervical cancer

Human papillomavirus (HPV) is the major caused of cervical cancer that causes death of 311000 women in 2018. [1] Based on their capability in inducing cancer, the HPV viruses are divided into 2 groups, the high risk and the low-risk group.[2] Consisted of at least 9 HPV subtypes, those are HPV 6,11,16,18,31,32, 45,52, and 58, the high-risk group is associated with 90% of cervical cancer and 90% of genital warts.[3] Approximately 67% of cervical cancer globally was caused by HPV16 and 18.[4] The main strategy to prevent HPV infection recommended by WHO is by vaccination. Nowadays there are three kinds of commercial prophylactic HPV vaccines, that are Gardsil, a quadrivalent vaccine containing HPV 6,11,16,18 antigens, Gardasil 9, a 9-valent vaccine containing HPV 6,11,16,18,31,33,45,52,58 antigens and a bivalent vaccine against HPV16 and 18 antigens.[3] The WHO recommends that the commercial HPV vaccine must cover at least two kinds of HPV subtypes, HPV 16 and HPV18 antigens.[5]

HPV is an unenveloped virus that has a circular double-stranded DNA genome of approximately 8 kb in size. [6] HPV has a capsid that formed by HPV major and minor capsid protein, which are Late (L) 1 and L2 proteins respectively. Naturally, L1 without the presence of other HPV structural and non-structural proteins has the capability to self-assemble to form virus-like particles (VLP).[7] Capsid proteins contain conformational epitopes that induce neutralizing antibodies. [8] Capsid proteins contain positively charged amino acids causing DNA that has negative charge can be assembled into VLP.[9] Based on its natural unique properties, many studies have explored the potentiality of L1 protein to be developed as vaccine, pseudovirion, and vehicle to deliver immunomodulator, adjuvant, DNA and proteins. [7,8,9]

Infection of HPV will be followed by serologic immune responses that mainly directed against conformational epitopes of viral capsid proteins, and this response will persist for many years.[9] Immunization of VLP will induce immune responses that resembling HPV infection because VLP presenting neutralizing antibody epitopes that are similar to those presented on the virus.[8] Thus, unlike a virus, VLP does not contain viral genome so the expression of proteins inducing cancers such as E6 or E7 can be avoided. Nowadays, in vaccine industries, there are 2 technologies used to produce L1 HPV vaccine, which is a yeast-expression (*Saccharomyces cerevisiae*) and Baculovirus-expression system. [8] By using these techniques, the mass of production of HPV vaccine becomes possible. The other promising expression system that could be developed as a production host of L1 protein is prokaryote. [11, 3]

Due to the limitation in providing a susceptible cell culture that can be infected by HPV, the capability of the antibody to prevent HPV infection was measured by using pseudovirion. Pseudovirion is L1 VLP that assemble DNA coding a certain reporter protein, such as green fluorescent protein (GFP). The production of pseudovirion is conducted by co transfecting plasmid coding L1 and L2 and plasmid coding reporter genes in 293 TT cell culture. L1 and L2 protein will be produced and assembled into VLP, and during the formation of VLP the DNA coding reporter gene will incorporate to VLP. The corporation of DNA to VLP is through the interaction of negative charge of the DNA with positive charges of the L1 amino acid. Like in HPV infection, pseudovirion enters cells via the interaction of VLP with HPV receptor expressed on 29TT cell, Heparan sulfate.[12] In neutralization assay, in the absence of neutralizing antibody, pseudovirion can enter cell culture, 293TT, and the reporter protein will be expressed. On the other hand, in the presence of neutralizing antibodies, the pseudovirion will be neutralized and cannot enter the cell causing reporter protein will not be expressed.

Beside beneficial in medicine, VLP also useful in nanotechnology.[13] SpyTag-Spy catcher technology or protein In nanotechnology, VLP can be decorated with a different antigen or protein. Decorating VLPs with target-antigens by genetic fusion of chemical modification is time-consuming and often leads to L1 protein miss folding or miss assembly. Some studies have been conducted to establish a platform for irreversibly decorating VLP simply by mixing with a protein of interest. One of the technologies used to develop irreversible decoration of VLP is Sphycatcher and SpyTag. Sphycatcher is a genetically-encoded protein designed to spontaneously form a covalent bond to its peptide partner.[13] Generally, a protein that has the capability to form a highly organized supramolecular structure with unique biological properties is chosen to be a Spy catcher.[14] The protein of interest tagged with peptide SpyTag form an irreversible covalent bond to the spy catcher protein via a spontaneous isopeptide linkage to create a peptide interaction that resists force and harsh conditions.[15] Even though the usage of L1 HPV as Spycartcher has been reported yet, L1 HPV is potential to be developed as Spy catcher. L1 could assembly to form big, stable molecules. A peptide that mediates spy catcher and spy tag interaction can be inserted in one of 5 major loops of L1 protein.

In this study, we construct the plasmid containing gene coding L1 HPV subtype 16. Plasmid will be used in the production of HPV 16 L1 protein. The HPV 16 L1 protein will be used in many studies, such as in the development of spy taq-spy cather nanotechnology or producing specific antibodies that can be used for diagnostic or other serological assays for HPV 16. More over producing HPV 16 L1 will give valuable experiences for developing vaccine or pseudovirion for other high risk HPV. Technically, producing recombinant protein in a prokaryote is simpler than in yeast or Baculovirus. The production of recombinant protein in bacteria does not need the expensive facility. [1,23,24,25,26]

METHODS

L1 coding gene. L1 used in this study a sequence is obtained from a back translation of the consensus sequence of L1 protein using DNA2 and has been codon optimized in accordance with prokaryote expression system. The L1 consensus sequence was generated by picking up the most frequent amino acid present at each position in a L1 protein alignment. The sequence of L1 full length proteins were collected from Genbank with accession number : AD33259.1, AIQ82831.1, ACN91168.1, AIQ82846.1, AIQ82845.1, AIQ82844.1, AIQ82843.1, AIQ82842.1, AIQ82841.1, AIQ82840.1, AIQ82839.1, AIQ82838.1, AIQ82837.1, AIQ82836.1, AIQ82835.1, AIQ82834.1, AIQ82833.1, AIQ82832.1, AIQ82829.1, AIQ82830.1. The L1 gene containing an open reading frame (ORF) of L1 protein with length 1515 bp was synthesized in IDT Malaysia via local supplier. The gene was cloned into universal cloning vecor pUC 19, and plasmid containing L1 gene was name pUCL1col. The sequence of L1 gene and protein is confidential because a patent is currently being filed.

Subcloned gen coding HPV 16 L1 protein into pQE80L. Gene coding HPV 16 L1 was subcloned from pUCL1col (obtained from PRVKP FKUI RSCM) to pQE80L (Qiagen) inserted in BamHI and HindIII fragment. Vector and insert used in the subcloned reaction were prepared by restricted 10 µg plasmid of each pQE80L and pUCL1col. The restriction was performed by adding 10 µg in a tube containing 1x Neb2 buffer, 1xBSA (NEB) and 40.000 Unit BamHI (NEB) and DNAseRNAse free water (Ambion) to volume 100µl. The mixture was incubated for 4 hours at 37°C. After that, the DNA was desalted using QiaecII gel extraction kit (Qiagen) following the procedure described by the manufacturer. Further, DNA was restricted by HindIII. DNA that has been cut with BamHI was added to a tube containing 1x Neb4 buffer, 1xBSA, and 40.000 Unit HindIII (NEB) and DNAseRNAse free water (Ambion) to volume 100µl. After incubated overnight at 37°C, DNA was run on 0.8% LMA containing crystal violet (Invitrogen). DNA was purified from LMA by using S.N.A.P UV-free DNA isolation kit (Invitrogen). Ligation was performed by using vector: insert comparison = 1:3. Ligation reaction is 80 ng vector, 80 ng insert, 1x ligation buffer (NEB), 2.5 Unit T4 Ligase (NEB) and water to volume 20 µl. reaction ligation was incubated at 16°C for overnight. Ligation was transformed into chemically competent Escherichia coli Top10[16] by the heat shock method.

Modeling Ribo Nucleic acid (RNA) secondary structure

The secondary structure of messenger RNA is generated from the HPV 16 L1 sequence (the sequence is unpublished) using the dynamic programming algorithm described in MaxExpect program.[17] The software can be accessed freely at Mathews Lab Home – University of Rochester (https://rna.urmc.rochester.edu)

Selection of recombinant cloned

Recombinant bacteria containing recombinant plasmid coding HPV 16 L1 was screened using PCR colonies. Primers pQEF (GTATCACGAGGCCCTTTCGTCT) and pQER (CATTACTGGATCTATCAACAGGAG) that recognizing specific sites in pQE80L was used to amplify the DNA inserted in multiple cloning sites. The PCR reaction was performed using DreamTaq DNA polymerase (Thermoscientifict) following the manufacturer's instruction (Thermoscientifict). Bacterial colonies were picked up using sterile toothpick strikes on replica plates and put into PCR tubes as PCR template. The colonies producing expected DNA amplicons were grown in 4 ml LB broth containing 100 µg/ml ampicillin, incubated at 37°C overnight. Plasmids were isolated using Miniprep (Qiagen) and characterized using enzyme restriction.[16] Further plasmid was sent to IDT Malaysia via local supplier for sequencing by using Sanger Method to verify the gene sequence. The shipment of DNA was accompanied by Material transfer agreement (MTA) signed by sender and receiver. MTA stated that the receiver was allowed to use the DNA only for method stated in research protocol.

Protein expression

The verified plasmid was transformed into bacteria *Escherichia coli* BL21 (DE3) chemically competence using heat shock transformation.[23] Three colonies were picked up and cultured in 4 ml LB broth containing 100 µg/ml ampicillin, incubated overnight at 37°C. One part of overnight culture was grown in 20 part of Terrific broth (Gliserol 1%, Trypton 1,2%, Yeast extract 1,2%, KH2PO4 0,34%, K2HPO4 (1,1%) containing 100 µg/ml ampicillin, after incubated for 1.5 hours, the cultures were incubated on ice for 15 minutes, and IPTG with various final concentration (0,1; 0,2 dan 1 mM) was added, and 1 ml cultures were collected after 3, 6 and overnight after induction. The incubation temperature of induction was 37°C.

Protein purification

Bacteria were lysed under native and denature conditions. In native condition, bacteria were lysed using buffer A (500mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 20%Sucrose, 1mg/ml Lyzosime. Under denature condition, bacteria were lysed using denature buffer (50mM Sodium Phosphate, 6 M Guanidine HCl, 300mM NaCl (pH8)). After incubated for 1 hour 20 minutes on rocking incubator 60 rpm (BioRad) at room temperature. After incubates, samples were centrifuged at 12000 rpm for 5 minutes at 4oC. Protein was purified using TALON® (Clontech) and the purification was conducted following the manufacturing instruction. Wash buffer used here was 45 mM Sodium Fosfat, 300mM NaCl, 5,4 M Urea, 10 mM imidazole (pH 8,0). Recombinant protein was eluted using 45 mM Sodium Fosfat, dan 300mM NaCl, 5,4 M Urea, 150/200 mM imidazole (pH 8,0). The purified recombinant protein was analyzed by run it on 12% SDS PAGE.

RESULTS

Linearized vector and L1 gene insert were successfully isolated from LMA, 4800 and 1515 bp respectively (Figure 1). After ligated recombinant plasmids were transformed into *E.coli* Top10, the growing colonies were subjected to PCR colony. The colonies produced 1840 bp amplicon indicated those colonies possibly contain the interested DNA. The pQE80L Wild type (WT) produced 289 bp DNA that closely migrated to 300 bp marker (Figure 2).

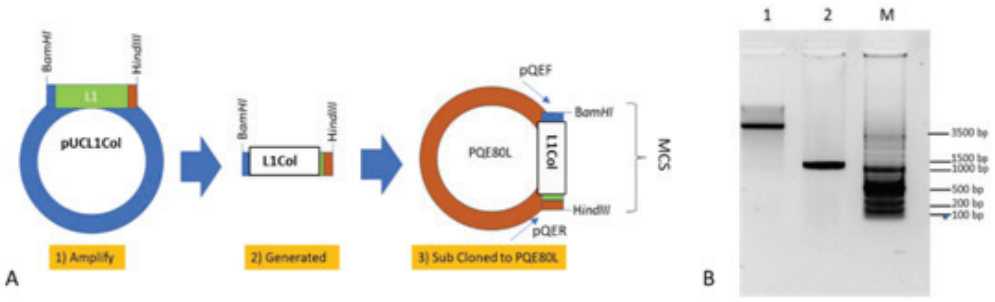

Figure 1. Schema of Subcloning plan (A) and vector (line 1) and insert (line 2) (B)

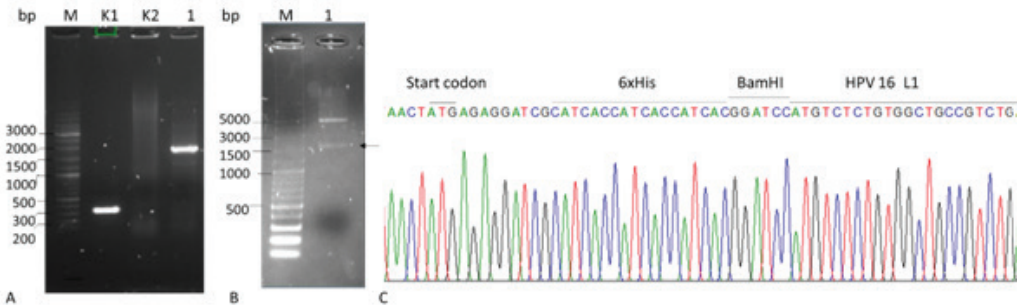

Figure 2. Screening of Recombinant plasmid. (A) PCR colony. M : Marker, K1 pQE80L WT, K2: Negative control, 1 pQE80L L1. (B) Restriction enzyme analysis pQE80L L1 with BamHI and HindIII, the arrow showed the fragment of L1 gene. (C). Sequencing result showed the position of L1 gene that was in frame with 6xHis.

Plasmids were isolated from PCR confirmed colonies. Once run on 0.8% agarose gel, recombinant plasmids migrated slower than pQE80L WT, indicating the size of those plasmids due to the insertion of L1 gene became larger than pQE80L WT (data was not shown). The recombinant plasmids were further analyzed using restriction enzyme. The recombinant plasmids produced DNA fragments 4800 and 1515 bp in length (Figure 2). The sequencing showed the inserted-gene sequences were not mutated and can be expressed in frame with 6xHis Tag (the full sequence of L1 was not shown).

The verified recombinant plasmid was transformed in bacteria hopes that used for recombinant protein production, BL21 (DE3). After inducing with IPTG with various concentrations showed there was overexpression of protein that migrates between marker 55 and 70 kDa, indicating L1 recombinant proteins were successfully expressed. Based on SDS page analysis, the 0.1 mM IPTG could induce the recombinant L1 as efficient as other IPTG concentrations. The recombinant bacteria could be expressed 3 hours after the addition of IPTG. Prolonged induction time to overnight also induced unspecific host protein (Figure 4). Analysis of messenger RNA secondary structure showed the RBS (AGGAG) in a single-stranded structure, and the last base (G24) of start codon formed external closing pair with T91, however that interaction did not infer protein expression.

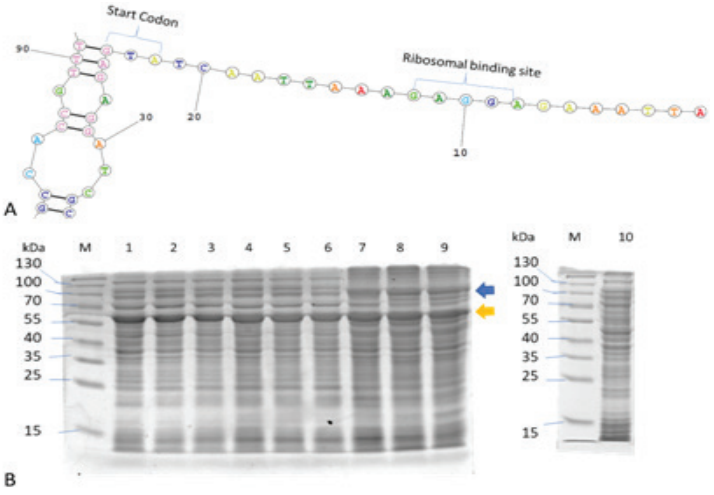

Figure 3. (A) The secondary structure modeling of 5'UTR L1-mRNA covering the ribosomal binding site and start codon generated by Maxexpect program. (B) The expression of L1 recombinant protein in E.coli BL21 (DE3) induced with various concentrations of IPTG and length of induction time. M: marker, 1-3 bacteria were induced for 3 hours at various concentrations of IPTG, no 4-6 were induced for 6 hours at various concentrations of IPTG, no 7-9 were induced for overnight at various concentrations of IPTG. The concentrations of IPTG: 0.1, 0.25 and 1 mM respectively. No 10: wild type BL21 (DE3). UTR: untranslated region.

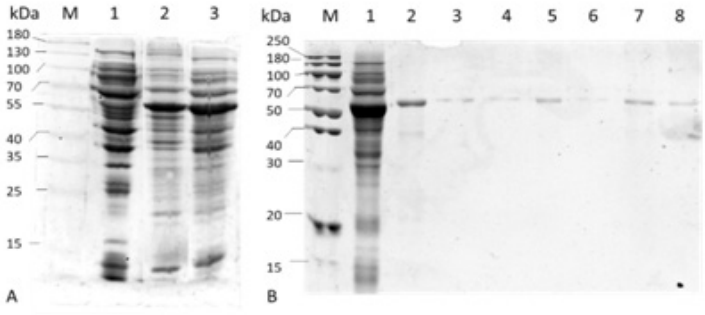

Figure 4. (A) Lysed bacteria M: Marker, 1: Supernatant 2: pellet 3: BL21 (DE3) expressing L1. (B) Purification of L1 recombinant using Talon, M: Marker, 1 BL21 (DE3) expressing L1, 2-6: the elution of L1 recombinant, 7-8: washed.

After lysed using native buffer, the recombinant L1 predominantly found in the pellet (Figure 4), indicating the protein retains in inclusion bodies. L1 recombinant protein fused with 6xHistidine Tag was successfully purified under denaturing condition using Talon^{R}. However, the protein also could be found in washing filtrate. Protein started to elute when incubated with elution buffer containing 150 mM Imidazole (Figure 4).

DISCUSSIONS

Bacteria containing plasmid coding gene of interest can be selected by combining some methods, such as PCR colonies, restriction profile of enzyme restriction of plasmid, a specific selectable marker, and sequencing. In this study, we used PCR colony as the 1st method to screen the bacteria bearing interested recombinant plasmid. In this study primer pair, pQEF and pQER, that recognized a specific region that flanks open reading frame of pQE80L was used in PCR colony. The amplification using pQEF and pQER causes the addition of 289 bp to the insert length (figure 2A) and the amplification of 289 pb in wild type palsmid (figure 2A). Specific primers that recognize gene of interest also can be used, but the possibility of the altering of primer annealing temperature after that gene was cloned into a vector must be taken into consideration. The second method to screen plasmid is based on the migration of plasmid on an agarose gel. The insertion of a gene into a plasmid causes the addition of plasmid size. Once run on agarose gel the recombinant plasmid will run slower than wild type. Further, the recombinant plasmid can be confirmed by DNA restriction pattern. Enzyme restriction has a specific target on DNA either plasmid or insert. In this study, the recombinant plasmid was cut with BamHI and HindIII. Those enzymes locate at 5' and 3' terminal of pQE80L cloning sites. The restriction of recombinant plasmid using BamHI and HindIII causes the separation of vector, 4800 bp, and insert 1515 bp (figure 2B). The sequence of interesting genes was confirmed using sequencing. By using proper software, the amino acid sequence of the inserted gene can be analyzed whether it in frame with 6xHistidine Tag or not. In this study, the L1 HPV 16 gen was cloned in frame with 6x His (figure 2C). L1 HPV16 recombinant can be expressed in prokaryote system that showed by the overexpression of 56 kDa protein band after induction with IPTG, whereas that band could not be found in wild type. The L1 gene used here was codon optimized in accordance to prokaryote to minimize the hindering of protein translation due to codon bias [11]. Based on mRNA modelling, the ribosomal binding site was in a single-stranded or un structure. Modelling was used to predict the translation probability of a mRNA in host cells.[18,19] Protein production in *E.coli* depends on the capability of 16sRNA to bind to a specific segment of mRNA called Shine Dalgano or ribosomal binding site [20] and the interaction only occurs when the SD/RBS in a single-stranded or unstructured state.[21] The interaction of SD/RBS with 16sRNA will be followed by the interaction of ribosome-bound initiator tMet with start codon (ATG). Based on RNA modelling, the last base of HPV 16 L1 ATG interacted with T91. However, this interaction does not hinder protein translation. Predicted RNA structure that generated based on the full sequence of mRNA probable difference to those generated in living system.[22] During transcription and translation process in vivo the elongation of mRNA chain occurs simultaneously with protein synthesis. Ribosome complex has occupied SD and ATG before the mRNA elongation reaches certain base that pairing with ATG.[18] By using Talon (Clontech), the recombinant HPV 16 L1 could be purified under denature condition, because under native condition, the protein retained in pellet. Previously been reported, L1 protein expressed in E.coli retained in inclusion bodies.[23,24] High level protein expression, high temperature during expression, high concentration of IPTG, the usage of strong promoter, partially folded or misfolded protein often results in aggregation of the recombinant protein into inclusion bodies.[25] By using chaotropic agents such as Guanidine HCl and Urea, the recombinant protein in inclusion bodies can be solubilized.[26] On the other hand, the presence of chaotropic agents can denature the protein. The denatured recombinant protein can renature by dialysis or diluting in native buffer. [26, 27] Therefore, modification of L1 protein at N-terminal has been reported could solubilize L1, and such modification did not affect the capability of L1 to self-assembly.[3]

The L1 protein of HPV 16 can be produced in various expression system, and the currently HPV prophylaxis vaccines were produced in mammalian system, that are Baculovirus and Yeast.[11,24,28,29,30,31,32] Compared to those mammalian expression systems, E.coli has some advantages such as fast growth, easy gene manipulation, low production cost and

easy to scale up.[28] Thus this system is suitable for low-income countries where cervical cancer results in higher mortality, and this expression system has been used to produce a low-cost HPV vaccine by Xiamen Innovax Biotech, China (Huang et al, 2017).

The sources of L1 gene used to developed VLP are varying. The gene could be amplified from keratynocyte cell line W12 (28), synthesized based on L1 gene downloaded from Genbank[11], extracted from patients[30], or ordered from ATTC company.[33] L1 gene used in this research is synthetic gene that codes a consensus L1 protein that represents the majority amino acid presented in different L1 HPV 16 proteins. Consensus sequence can accommodate the diversity of L1 HPV 16.[34] Vaccine based on consensus sequence expectantly could induce strong and broad immune response.[34]

In conclusion, gene coding HPV 16 L1 has been successfully cloned into pQE 80L, a prokaryote expression system. The cloned gene can be expressed *E.coli* in BL21(DE3) and can be purified using Talon^{R}.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Increasing serum miR-124-3p expression is associated with the high survival rate of a rectal cancer patient after neoadjuvant chemoradiotherapy

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Abstrak

Latar Belakang: *Kanker kolorektal menempati urutan ketiga penyebab kanker di dunia, dengan prevalensi kanker rektum sebanyak 30% dari total kasus. Saat ini belum ada biomarker yang efektif untuk memprediksi respon pasien terhadap terapi yang diberikan. Beberapa penelitian menggunakan potensi miRNA sebagai biomarker untuk melihat respon terapi. Salah satunya yaitu MiR-124-3p berperan sebagai tumor supresor yang mengalami penurunan ekspresi pada berbagi jenis kanker. Tujuan dari penelitian ini adalah untuk meneliti ekspresi miR-124-3p dari pasien kanker rektum yang menerima nCRT, dan menganalisis hubungannya dengan kelangsungan hidup pasien dan parameter klinis lainnya.*

Metode: *Penelitian ini melibatkan 15 orang pasien yang didiagnosis menderita kanker rektum lokal dan menjalani kemoradioterapi neoajuvan (radioterapi 45-50 Gy dengan fraksi 1,8-2 Gy selama 1-3 bulan, dan kemoterapi 5-fluororacil secara oral). Sampel penelitian berupa darah intravena sebanyak 5 ml diambil saat sebelum dan sesudah kemoradioterapi. Selanjutnya ekspresi miR-124-3p dianalisis menggunakan qRT-PCR dan dikalkulasi menggunakan metode Livak.*

Hasil: *Terdapat hubungan signifikan antara peningkatan ekspresi miR-124-3p dengan sintasan hidup pasien (P=0,003; OR =30, 95% CI = 1,41 – 638,15), serta adanya peningkatan ekspresi miR-124-3p yang signifikan (P<0,041, fold change sebelum=1,14 ± 1,25; sesudah=2,4 ± 1,84) setelah dilakukan kemoradioterapi.*

Kesimpulan: *Hasil ini mengindikasikan bahwa miR-124-3p berpotensi menjadi biomarker untuk memprediksi sintasan hidup pasien kanker rektum yang menerima kemoradioterapi.* ***(Health Science Journal of Indonesia 2019;10(2):90-5)***

Kata kunci: *kanker rektum, kemoradioterapi, miR-124-3p, sintasan hidup*

Abstract

Background: Colorectal cancer is the world's third most prevalent cancer, which 30% of cases are rectal cancer. Today, the effective diagnostic marker to accurately predict clinical outcome patients response to therapy did not found yet. Several research studies have indicated that miRNA potential as a prognostic biomarker. MiR-124-3p plays as tumor suppressor that significantly down-regulated in some cancer and could radiosensitize human colorectal cancer cells. The aim of the study is to investigate the expression of miR-124-3p from rectal cancer patient who receive nCRT, and analyze its association with patient survival and others clinical parameters.

Methods: This research involved 15 patients with histologically confirmed locally advanced rectal cancer (LARC) and received neoadjuvant chemotherapy/nCRT (radiotherapy 45-50 Gy with 1,8-2 Gy fractions over 1 to 3 months and chemotherapy 5-fluorouracil was administered orally). Patient blood (5 ml) were collected from peripheral venous before and after neoadjuvant chemoradiotherapy. miR-124-3p expression was performed using qRT-PCR and calculate using Livak method.

Results: In this study, we found that increasing of miR-124 was significantly associate with high survival of rectal cancer patient (P = 0,003; OR =30, 95% CI = 1,41 – 638,15). Average of miR-124-3p expression increase significantly after nCRT (P<0,041, fold change before=1,14 ± 1,25; after=2,4 ± 1,84).

Conclusion: Our finding suggests that miR-124-3p expression in blood serum was potential as biomarkers to predict rectal cancer patient survival after neoajduvant chemoradiotherapy. ***(Health Science Journal of Indonesia 2019;10(2):90-5)***

Keywords : rectal cancer, chemoradiotherapy, miR-124-3p, survival

Colorectal cancer is the world's third most prevalent cancer and causing death due to cancer in the second rank. Rectal cancer occurs approximately 30% of all colorectal cancer cases and has higher reccurance and resistance to therapy than colon cancer.[1,2] The standard treatment rectal cancer is called trimodality therapy, consist of neoadjuvant chemoradiotherapy (nCRT), then followed by a total mesorectal excision and chemotherapy. Organization of nCRT can essentially increment patient survival, however local reccurence is still common due to resistance. Approximately 40–60% of rectal cancer patients who treated with nCRT achieve improvement in the pathological reaction. Although about 40% of patients still recur and some of them were unable to survive.[2,3] The overall 5-years survival rate for people with rectal cancer is 67%. However, colorectal cancer survival rates may differ based on several factors.[3] Today, many molecular mechanisms underlying sensitivity or resistance to chemoradiotherapy have been discovered and developed to accurately predict clinical result and response to rectal cancer treatment. MicroRNA (miRNA) is one of molecular factor that closely associates to tumor chemoradiotherapy sensitivity and appears great prospects of research and clinical application.

MiRNA is small endogenous non-coding RNA (19-25 nucleotides) that regulates post-transcription gene expression by partly binds complementary sequences of its target messenger RNA (mRNA). This binding results in degradation and/or translation inhibition and leads to reduced protein expression. miRNA can function as an oncogenic (onco-miR) or tumor-suppressive (tumor-suppressor miR).[4] During the growth of cancer, miRNA expression is frequently deregulated in many types of cancer and cause abnormal level of expression that related with cancer clinicopathological characteristics. Therefore, the expression modifications of cancer-associated miRNA emerge as promising diagnostic markers that correlate with cancer development of cancer, patient survival, sensitivity and resistance to therapy.[5]

MiR-124-3p or called miR-124 acts as a tumor suppressor that significantly down-regulated in many human malignant tumors including breast cancer, malignant glioma, gastric cancer, and colorectal cancer.[6-9] Recent studies have shown that miR-124-3p can radio sensitizes human colorectal cancer cells through PRRX1 targerting.[9] MiR-124-3p may also be a prospective marker for gastric cancer and associated with poor prognosis in colorectal cancer. Increasing of miR-124-3p expression in colorectal cancer could inhibit colorectal cancer cells development.[8,10]

MiRNA can be secreted by cells and circulate in a stable phase in the human blood. Blood samples are easy to acquire and suitable for clinical use. Circulating miRNA are a new class of non-invasive biomarkers that show excellent stability under a multitude of physical and chemical circumtances.[11] The aim of this study is investigating the expression of miR-124-3p from blood serum in a rectal cancer patients who receive nCRT, and analyze its association with patient survival and other clinical parameters. Serum samples are much easier to obtain and simpler to access than cancer tissues. Serum based miRNAs can provide clues in rectal cancer therapy surveillance.

METHODS

Patients and sample collection

This Cohort study involved 15 patients with histologically confirmed locally advanced rectal cancer (LARC) with clinical TNM stage II and III, no history of previous malignancy based on MRI and histopathology examination. All patients received neoadjuvant chemoradiotherapy in Kariadi Hospital, Central Java, Indonesia, between 2017-early 2018. All patients received neoadjuvant chemoradiotherapy with 1,8-2 Gy fractions over 1 to 3 months depending on radiotherapy 45-50 Gy and 5-fluorouracil chemotherapy orally.

Patient blood (5 ml) were collected from peripheral venous, before and after neoadjuvant chemoradiation. By centrifugation at 3000 rpm for 15 minutes, serum was isolated from the blood. Serum samples were separated into 3 aliquots and stored at -80°C until further analysis. The characteristics of patients are shown in Table 1. This research was approved by the Medical Faculty of Diponegoro University and Kariadi Hospital Ethics Committee number 14/EC/FK-RSDK/I/2017, then continued by the Medical, Public Health and Nursing Faculty Ethics Committee, Universitas Gadjah Mada (Ref : KE/FK/1008/EC/2018), in accordance with the Declaration of Helsinki. All respondents acquired informed consent prior to enrollment in the research.

RNA isolation and cDNA synthesis

Total RNA was isolated from 200 µl of blood serum using miRCURY™ RNA Isolation Kit – Biofluids

(Qiagen), following the manufacture's guidelines and specific application instruction. RNA concentration and purity were controlled by UV spectrophotometry using Nanodrop (NanoVue Plus, GE Healthcare, Life Science). Total RNA was eluted in RNAse-free water and stored at -80^{0}C until use. cDNA was synthesized using the reverse transcription reaction miRCURY LNA RT Kit (Exiqon) and performed using thermal cycler (Applied Biosystems^{TM} A24811) following the manufacture's instructions and stored at -20^{0}C until use.

Quantitative real-time PCR (qRT-PCR)

Analysis of qRT-PCR was performed using a SYBR-green-containing PCR (miRCURY LNA SYBR Green PCR Kit, Qiagen) and samples were run on CFX96 Touch^{TM} Real-Time PCR Detection System (Bio-Rad, USA) in a total volume 10 µl. U6 small non-coding RNA was used as a reference gene. The primer sequence for miR-124-3p was 5'-UAAGGCACGCGGUGAAUGCC-3' and U6 was 5′-CGCTTCGGCAGCACA TATACTA-3′ (miRCURY LNA miRNA PCR Assay, Qiagen). All these samples were standardized to reference gene and fold changes were calculated using the Livak's method relative quantification ($2^{-\Delta\Delta Ct}$).

Statistical methods

Categorical data are shown as the frequency counts and percentages, while continuous variables are shown as the mean plus or minus standard deviation. The association between miR-124-3p expression and patient's characteristic was assessed using χ^2 test (Chi-square, Fisher's exact test), while Odds Ratio (OR) equivalent to the 95% CI was used to evaluate the power of the associations. Significant differences in miR-124-3p expression before and after neoadjuvant chemoradiation were determined using Wilcoxon's test. A statistically significant difference was regarded to show P-value < 0,05.

RESULTS

Patient clinicopathological characteristics

Table 1 shows the main clinicopathological characteristics of 15 patients included in the study. Their median age was 46 years (range 26-65 years) and dominated by male. More than half of patients had well differentiated histopathology and had clinical stage III. Early CEA level average is 33,9 ng/mL, higher than the recommended normal level (5 ng/mL). Eleven patient from 2017 until early 2018 who still alive until early 2019, were grouped as the patient who has high survival.

Table 1. Patient clinicopathological characteristic

Patient Characteristics	
Sex, n (%)	
Male	10 (67)
Female	5 (33)
Age (years)	
Mean ± SD (min-max)	46,1 ± 12,1 (26-65)
Differentiation, n (%)	
Well	11 (74)
Moderate	2 (13)
Poor	2 (13)
Early T Stages, n (%)	
T1	1 (7)
T2	2 (13)
T3	6 (40)
T4	6 (40)
Early N Stages, n (%)	
N0	2 (13)
N1	9 (60)
N2	4 (27)
Early Clinical TNM Stage, n (%)	
II	2 (13)
III	13 (87)
Early CEA level (ng/mL)	
Mean ± SD (min-max)	33,9 ± 68, 4 (0,04 – 200)

SD=standard deviation; n=amount of subject; T=tumor; N=node; TNM=Tumor size, Node status, Metastasis; CEA=carcino embryonic antigen; min=minimal; max=maximum

Relationship between miR-124-3p expression and patient clinicopathological characteristics

The relationship between miR-124-3p expression and clinicopathological characteristics is shown in Table 2. Increasing of miR-124-3p expression during nCRT were significantly associated with patient survival (P < 0,05; OR =30, 95% CI = 1,41-638,15). No significant relationship was observed between the miR-124-3p and sex, age, differentiation, early T stage, early N stage, early clinical TNM stage, down staging and early CEA level.

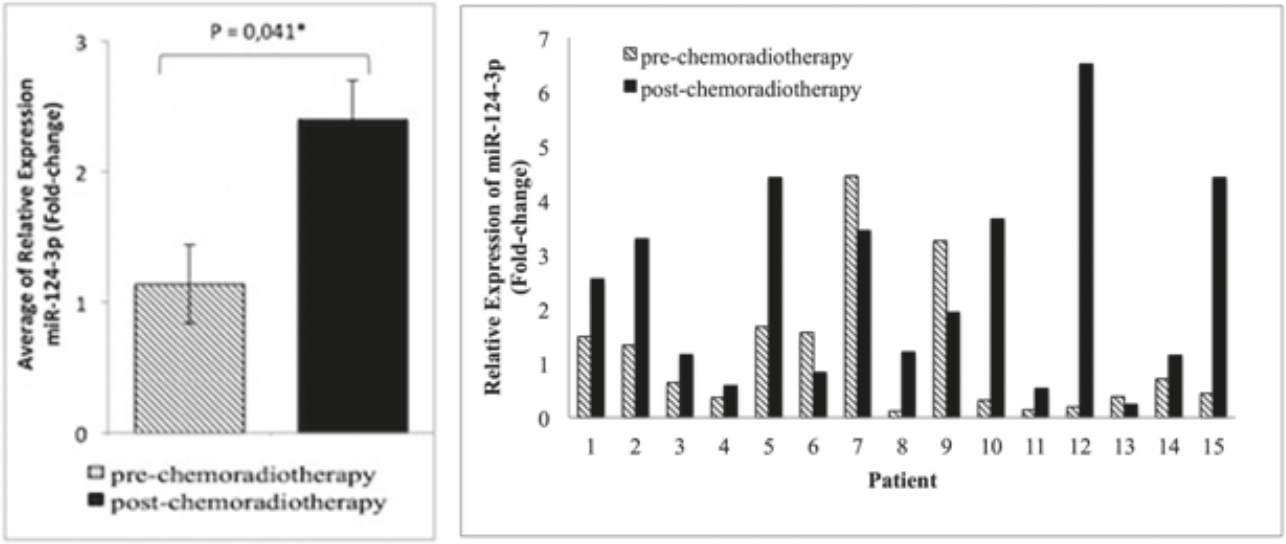
Average difference of miR-124-3p expression

Average difference of miR-124-3p expression and each patient are shown in Figure 1. MiR-124-3p expression increase significantly after nCRT (P<0,05, before=1,14 ± 1,25; after=2,4 ± 1,84; delta=1,26 fold). MiR-124-3p expression increase in 11 patients (73%), which 10 patients could survive. During follow-up, 4 patients died of rectal cancer because of worse prognosis (patient no. 5, 6, 9, 13). Three of them have decrease miR-124-3p expression.

Table 2. Association of miR-124-3p and patient characteristic

Patient Characteristic	Mir-124-3p Expression After Chemoradiation, n(%)		OR (95% CI)	P
	Increase	Decrease		
Sex				
Male	7 (63,6)	3 (75)	0,58 (0,04 - 7,6)	0,593
Female	4 (36,4)	1 (25)		
Age				
< 50	7 (63,6)	2 (50)	1,75 (0,17 - 17,69)	0,538
≥ 50	4 (36,4)	2 (50)		
Differentiation				
Well	8 (72,7)	2 (50)	2,67(0,25 - 28,44)	0,407
Moderate - Poor	3 (27,3)	2 (50)		
Early T Stages				
T1 - T2	3 (27,3)	0 (0)	1,5 (1 - 2,24)	0,363
T3 - T4	8 (72,7)	4 (100)		
Early N Stages				
N0	1 (9,1)	1 (25)	0,3 (0,14 - 6,38)	0,476
N1 - N2	10 (90,9)	3 (75)		
Early Clinical TNM Stage				
II	1 (9,1)	1 (25)	0,3 (0,01 - 6,38)	0,476
III	10 (90,9)	3 (75)		
Down staging				
Yes	9 (81,8)	4 (100)	0,69 (0,48 - 0,99)	0,542
No	2 (18,2)	0 (0)		
Early CEA level				
Normal (≤ 5 ng/mL)	3 (42,9)	1 (33,3)	1,5 (0,09 - 25,39)	0,667
Elevated (> 5 ng/mL)	4 (57,1)	2 (66,7)		
Survival				
Survive	10 (90,9)	1 (25)	30 (1,41 - 638,15)	0,033*
Died	1 (9,1)	3 (75)		

TNM=Tumor size, Node status, Metastasis; CEA=carcino embryonic antigen
Chi-square test (Fisher's exact test) *P-value significant < 0,05



Wilcoxon's test, P significant < 0,05

Figure 1. Difference of average miR-124-3p expression before and after nCRT (left); difference of miR-124-3p expression in each patients (right).

DISCUSSION

In rectal cancer clinical management, a recurrence and resistance after therapy is a challenging obstacle. Cancer generally more heterogeneous during carcinogenesis influenced by complex mechanism and affect to varies therapy result. Independent variables in clinical practice, such as pathological subtype, histological type, clinical stage, lymph node status and interval, may affect the prognosis of LARC patients receiving chemoradiotherapy. Some molecular factors can also influence the prognosis and sensitivity LARC patients to chemoradiotherapy due to genetic, transcriptomic, epigenetic and/or phenotypic changes.[12] This study focuses mainly on epigenetic, particularly microRNA.

Chemoradiotherapy resistance is an important factor that influences the prognosis of LARC. Therefore, predictive biomarkers capable of predicting sensitivity to chemoradiotherapy urgently need to help identify patients who would actually benefit from chemoradiotherapy. Chemoradio-resistance is a complicated process, involving cancer stem cell or tumor-initiating cells (TICs), angiogenesis which promotes by tumor-associated macrophages (TAMs), overexpression of DNA repair protein and autophagy.[13-15] Some earlier study has shown that miRNAs are associated with tumor cell chemoradio-sensitivity. MiRNA overexpression in tumor cells may increase or decrease chemoradio-sensitivity. Some miRNA expression has been down-regulated in resistant patients, but after up-regulation or administration, it may improve the impact of chemoradiotherapy.[16,17]

In this research, we examine the difference of miR-124-3p expression in the LARC patient serum before and after neoadjuvant chemoradiotherapy, and focusing on its relationship with patient survival for 1-2 years after treatment. Notably, more than half of patients who can survive after neoadjuvant chemoradiotherapy have an increase miR-124-3p expression and both variable significantly associated ($P < 0,05$; OR =30, 95% CI = 1,41 – 638,15). Although interesting, the limitation of this study obtained in small sample size of patients with cancer treated, so we suppose that a more precise result will be determined by a massive measurement of subjects for the next study.

In earlier study, some radiation resistance model of cell line was developed and miRNA-mediated molecular mechanism was investigated. MiR-124-3p in the radiation-resistant cell line was found to be considerably down-regulated. Down-regulation of miR-124-3p is linked with metastasis and poor prognosis in colorectal cancer.[18] Whereas miR-124-3p over-expression has been able to sensitize radiation-resistant cells by reducing the fraction of cell survival and viability[19,20]. It can be used as independent prognostic factor in colorectal cancer patient.[21]

MiR-124-3p has been involved as modulator of colorectal cancer carcinogenesis in many past studies. Some targets of miR-124-3p were identified in the regulation of colorectal cancer. MiR-124-3p regulates colorectal cancer growth by targeting PKM (pyruvate kinase muscle) gene that plays a role inhibit glycolysis rate in Warburg effect and also targets PRPS1 and RPIA gene that reduces pentose phosphate pathway and proliferation.[22,23] MiR-124-3p enhance radiotherapy sensitivity by targeting PRRX1, which act as epithelial-mesenchymal transition (EMT) inducer and stemness regulator and directly targets DNA methyltransferase 3B and DNMT1 in a colorectal cancer cell. miR-124-3p have multiple effects and targets in the development of colorectal cancer, become very potential for therapeutic target in the future.[9,24]

In conclusion, we identified that the average of miR-124-3p expression in rectal cancer patient with good prognosis was increased after neoadjuvant chemoradiotherapy and associate with high survival rate of patient. MiR-124-3p was found high-abundance and easy to identify in serum. Our study showed that miR-124-3p expression in blood serum was potential as biomarkers to predict the survival of rectal cancer patients after neoadjuvant chemoradiotherapy.

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The effect of ethanolic leaves extract of soursop (*Annona muricata* L.) on human colorectal cancer cell line: cell viability and in silico study to cyclin D1 protein

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Abstrak

Latar Belakang: *Kanker kolorektal merupakan transformasi patologis dari epitel kolon dan rektum normal menjadi massa jaringan abnormal, perubahan ini terjadi karena ekspresi berlebih dari protein cyclin D1 yang menginduksi proliferasi sel kolorektal secara berlebihan. Pengobatan dan pencegahan kanker kolorektal dapat dilakukan secara alami dengan mengonsumsi ekstrak daun Annona muricata L. (sirsak). Sirsak dikenal karena banyak komponen fitokimia yang berfungsi sebagai anti kanker.*

Metode: *Penelitian ini menggunakan sel kanker kolorektal HT-29 yang diberi ekstrak etanol daun sirsak dan 5-Fluorourasil (5-FU). Tujuannya untuk menemukan konsentrasi sitotoksisitas yang dapat menghambat 50% populasi sel HT-29 (CC50) dan konsentrasi yang didapat sebelumnya akan diuji dengan metode uji MTT. Analisis docking molekuler dilakukan antara molekul-molekul dari ekstrak etanol daun sirsak terhadap protein Cyclin D1 menggunakan perangkat lunak molecular operating environment (MOE) 2013.08.*

Hasil: *CC50 ekstrak etanol daun sirsak adalah 278* µg */ mL dan 5-FU adalah 88* µg */ mL. Persentase terendah sel HT-29 yang layak adalah 2 x CC50 setelah perlakuan ekstrak etanol daun sirsak (40,4 ± 1,3%) dibandingkan dengan 5-FU (52,8 ± 4,3%), kontrol pelarut (97,2 ± 1,4%), dan kontrol sel (100%). Analisis docking molekuler untuk protein cyclin D1 diperoleh asam N-hexadecanoic dan molekul phytol sebagai kandidat yang baik untuk menghambat protein cyclin D1.*

Kesimpulan: *Ekstrak etanol daun sirsak dapat menurunkan viabilitas sel kultur kanker kolon HT-29 dan berdasarkan analisis molekular docking dilihat dari energi bebas gibbs (ΔG) dan afinitas tertinggi (pKi) diperoleh N-hexadecanoic dan molekul phytol sebagai penghambat protein cyclin D1.* ***(Health Science Journal of Indonesia 2019;10(2):96-102)***

Kata Kunci*: Kanker kolorektal HT-29, ekstrak etanol daun sirsak, viabilitas sel, molecular docking, cyclin D1*

Abstract

Introduction: Colorectal cancer is a pathological transformation of normal colon and rectum epithelial that becomes an abnormal tissue mass, due to the overexpression of cyclin D1 protein that inducing excessive proliferation of colorectal cell. The treatment and prevention of colorectal cancer could be done naturally by consuming leaves extract of Annona muricata L. (soursop). Soursop is known for many phytochemical components that serve as an anti-cancer.

Methods: This study was used HT-29 colorectal cancer cell that treated with ethanolic leaves extract of soursop and 5-Fluorourasil (5-FU) to find the cytotoxicity concentration that can inhibit 50% of HT-29 cell population (CC_{50}) and the next concentrations of them were treated for next treatment with MTT assay. Molecular docking analysis of the compounds of ethanolic leaves extract of soursop to cyclin D1 protein used molecular operating environment (MOE) 2013.08 software.

Results: CC_{50} of ethanolic leaves extracts of soursop was 278 µg/mL dan 5-FU was 88 µg/mL. The lowest percentage of viable HT-29 cell was 2 x CC_{50} after ethanolic leaves extract of soursop treatment (40,4±1,3%) was compared to 5-FU (52,8±4,3%), solvent control (97,2±1,4%), and cells control (100%). Analysis of molecular docking to cyclin D1 protein was obtained N-hexadecanoic acid and phytol molecules as good candidates to inhibit cyclin D1 protein.

Conclusions: The ethanolic leaves extract of soursop could be a good alternative treatment for colorectal cancer and its compounds had ability to inhibit cyclin D1 protein (the highest gibbs free energy (ΔG) and affinity (pKi)). ***(Health Science Journal of Indonesia 2019;10(2):96-102)***

Keywords: Colorectal cancer, ethanolic leaves extract of soursop, cell viability, molecular docking, cyclin D1

Colorectal cancer is a pathological change in the normal colon and rectal tissue to an abnormal tissue caused by genetic and environmental changes. The 'rise' of colorectal cancer can be attributed to the increasingly aging population, modern dietary habits and an increase in risk factors such as smoking, low physical exercise and obesity.[1] According to the International Agency for Research on Cancer (IARC), the incidence of male colorectal cancer in the world is the third largest case (21%) after lung cancer and prostate cancer, while the incidence of colorectal cancer in women in the world is the second largest case (14%) after breast cancer.[2] Colorectal cancer therapy used is surgery, radiotherapy and chemotherapy.[3] This is considered to be less effective because of side effects, so the alternative therapy is needed, such as consuming *Annona muricata* L. (soursop).[4,5]

Annona muricata L. is a type of tropical plant known for containing many phytochemical components such as alkaloids, annonaceous acetogenin, megastigman, flavonol triglycosides, phenolics, and cyclopeptides found in leaves, fruits, seeds, and roots that can act as anti-inflammatory, anti-inflammatory infection and anti-cancer.[6,7] Soursop leaves extract can produce cytotoxic effects on colorectal cancer cell cultures such as HT-29, HCT-116,[5] COLO-205,[8] and DLD-1.[9] Soursop leaves extract is also known to reduce the expression of cyclin D1 protein in phase G1/S.[5,10]

Cyclin D1 is a protein encoded by CCND1 gene and controls cell cycle especially at the G1 phase. In this process, the expression of cyclin D1 protein increases and binds to cyclin dependent kinase 4 or 6 (CDK4 / 6) protein to form active kinase. That complex can phosphorylate or inactivate the retinoblastoma (Rb) protein. Phosphorylated Rb causes the transcription factor E2 factor (E2F) to promote the transcription of genes needed for cell division.[11] In colorectal cancer, cyclin D1 can be a significant marker. Meta analysis of 21 studies on the prognostic value of cyclin D1 expression showed that high cyclin D1 levels were associated with poor overall survivor and cell line especially HT-29.[12,13] High regulation of cyclin D1 was detected at 527 of 557 (94,6%) tumor cases.[14] High expression of cyclin D1 causes abnormal cell cycle.[15] The aim of the study is to investigate the effect of ethanolic leaves extract of soursop on HT-29 colorectal cancer cell viability and molecular docking of its active composition on the cyclin D1 protein.

METHODS

Plant materials

Soursop leaves were extracted 96% ethanol and obtained from Indrawati, et al.[9] *Annona muricata* extract used in this study is a standardized vacuum dried extract produced by Javaplant, Central Java, Indonesia.

Cell culture

HT-29 (human colon cancer cells) were obtained from the American Type Cell Collection (ATCC, Manassas, VA, USA). The cells were maintained in high glucose-DMEM (Gibco™), 1% penicillin-streptomycin (Gibco™), 1% amphotericin B (Gibco™), and 10% fetal bovine serum (Gibco™) in a humidified atmosphere with 5% CO_2 in the air at 37°C.

Cell viability assay

Cell viability was evaluated by using the 3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay as previously described by Bahaguna A et al.[16] In brief, cells ($1x10^4$ cells/mL) were treated with ethanolic leaves extract of soursop, 5-Fluorouracil (5-FU) (Curacil®) as a standard anticancer drug was used as a positive control, dimethyl sulfoxide (DMSO) 100% (Sigma Aldrich) as a solvent control, and only complete medium as a negative control at different concentrations in 96-well plate and incubated for 24 h. The concentration of ethanolic leaves extract of soursop was 12,5-400 µg/mL, 5-FU was 25-100 µg/mL, and 0,1% DMSO. MTT assay was measured at 570 nm absorbance using a microplate reader (Promega™ Glomax™).

The anti-proliferative potential of the treatment is expressed as the value of the cytotoxicity concentration (CC_{50}), i.e. the concentrations that causes inhibition of 50% cell growth and is calculated based on the percentage of cell viability. The percentage of cell viability is ¼ (absorbance of treated cells / absorbance of untreated cells) x 100%. The CC_{50} of ethanolic leaves extract of soursop and 5-FU were obtained by the first MTT assay and followed by using concentrations of ½ x $CC_{50,}$ 1 x CC_{50}, and 2 x $CC_{50.}$

Molecular docking with cyclin D1 protein

Docking simulation of extract *Annona muricata* L. to cyclin D1 was started by ligand (compounds) and receptor (protein) preparation. The compounds were obtained from Gavamukulya Y, et al,[17] i.e 2-pentadecanol; oleyl alcohol; 1,2-benzenedicarboxylic acid, butyl octyl ester; 3,7,11,15-tetramethyl-2-hexadecen-1-ol; N-hexadecanoic acid; hexadecanoic acid, ethyl ester; phytol; 1,E-11,Z-13-octadecatriene; 7-tetradecenal, (Z); 9,12-octadecadienoic acid, ethyl ester; cis, cis, cis-7,10,13-hexadecatrienal; and

1,2-benzenedicarboxylic acid, diisooctyl ester by using GC-MS Analysis. These specific compounds have been analyzed from ethanolic leaves extract of soursop. The structure of the compounds was obtained from chemspider database (www.pubchem.com). The fasta sequences of cyclin D1 protein were obtained from database national center for biotechnology information (NCBI) and the 3-D structure was from http://swissmodel.expasy.org (Fig 1).

The ligand and protein were optimized by molecular operating environment (MOE) 2013.08 software. Geometry optimization and energy minimization of cyclin protein were carried out using the MOE software with PDB format. The structure of cyclin D1 protein was added to parameters such as hydrogen atoms, partial charges, and gas phases. The addition of hydrogen atoms and protonation was performed on cyclin D1 protein. Partial charges were regulated by using a partial charge. Protein energy was minimized by the Merck Molecular Forcefield 94x (MMFF94x) force field. The protein was performed on gas phase solvation with a fixed charge and optimized with a mean square root gradient (RMS) of 0.05 kcal/Åmol. The overall optimization file was obtained in the .moe format.

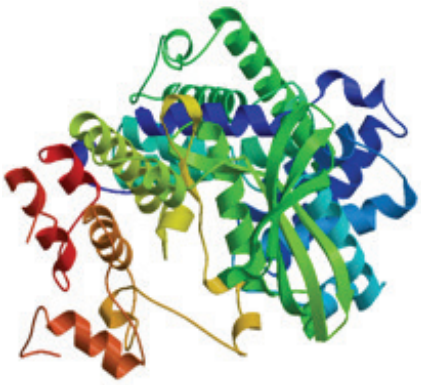

Figure 1. 3-D Cyclin D1 protein structure (2w96.1.A)

This protein has 100% homology with the template.

Geometry optimization and energy minimization of ligand structures using MOE software with the .mdb format. Ligand candidates were kept with the .mol format in the database viewer of MOE software. Ligands were washed with a computational program, the partial charge was adjusted and optimized using the MMFF94x force field. In addition, ligand energy was minimized using minimal energy with an RMS gradient of 0.001 kcal/ Åmol and the resulting file was saved in the .mdb format. Molecular docking of ligand molecules with cyclin D1 protein was carried out by a dock program simulation in MOE. Molecular docking used triangular matching by repeating energy readings for each position on cyclin D1 protein 100 times (retain: 100). The assessment function used London dG and refinement force. The last retained of refinement products were the most suitable conformation of each ligand molecules.

Molecular docking calculations were seen in the output format of the viewer.mdb. Several parameters of protein-ligand interactions can be analyzed, including bond free energy (ΔG) and affinity (pKi). The protein-ligand complex selected was the smallest bond energy value and the greatest bond affinity.

Statistical analysis

Cell viability values were presented as mean ± SEM from four different experiments. One-way variance analysis (ANOVA) was performed using SPSS v.21. Differences were considered significant at $p<0.05$.

Ethical Clearance

This study does not require ethics approval because it does not use animal or human subjects.

RESULTS AND DISCUSSION

Cell Viability Assay

One-way ANOVA statistical test and post-hoc LSD test shows the effect of exposure time of soursop leaves extract and positive control (5-FU) on HT-29 cell viability. The sign (*) shows a significant difference ($p <0,05$) and the sign (**) shows a significant difference ($p <0,01$).

The linear regression curve between the concentrations of the two test compounds obtained an equation for ethanolic leaves extract of soursop (y = -0,0363x + 60,078 and R2 = 0.8802) and 5-FU (y = -0.3067x + 77.052 and R2 = 0.9782). Equation showed CC_{50} was used to determine the concentration of ethanolic leaves extract of soursop and 5-FU which can inhibit half of the cell population. The CC_{50} value was obtained from the x variable by entering a value of 50 (the standard value was taken from 50% inhibition) to the y variable in the equations of the two tests. Based on the cytotoxic test of ethanolic leaves extract of soursop and 5-FU, showed that 5-FU had cytotoxic activity three times lower than ethanolic leaves extract of soursop, i.e. CC_{50} of soursop leaves extract was 278 µg/mL and CC_{50} of 5-FU was 88 µg/mL. R^2 showed the correlation of the concentration with a decrease in viable cell.

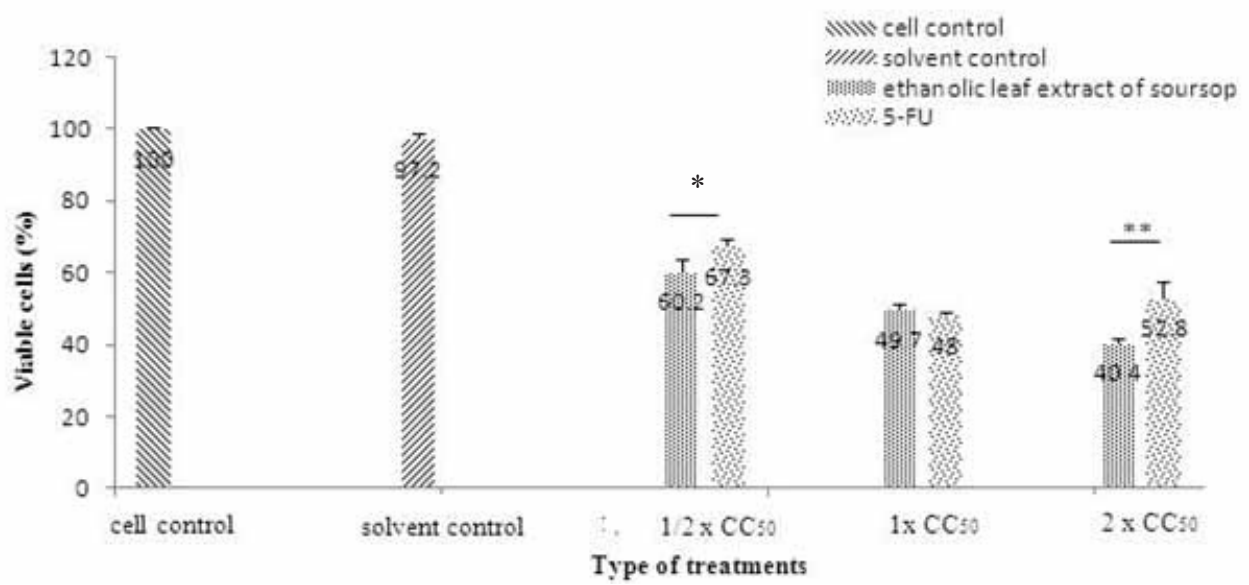

Figure 2. Cell viability of HT-29 after treatment with different CC50 concentration of ethanolic leaves extract of soursop and control. The sign (*) shows a significant difference (p <0,05) and the sign (**) shows a significant difference (p <0,01)

The treatment group between ethanolic leaves extract of soursop compared with 5-FU for a concentration of ½ x CC_{50} was statistically significantly different with a value of p = 0,001 (p <0.05). Soursop leaves ethanol extract showed cell viability of 7,1% lower compared to 5-FU. The ethanolic leaves extract of soursop compared to 5-FU for a concentration of 1 x CC_{50} did not have a statistically significant difference (p> 0,05) with a value of p = 0,509. The difference in viability of the two was only 1,7%, namely the 5-FU viability was lower than the ethanolic leaves extract of soursop. The ethanolic leaves extract of soursop compared to 5-FU for a concentration of 2 x CC_{50} had a very statistically significant difference with a p value <0,01. Soursop leaves ethanol extract had a cell viability of 12,4% lower compared to 5-FU (Fig 2).

Molecular Docking with Cyclin D1 Protein

Molecular docking analysis with MOE obtained two molecules that have the lowest ΔG and the highest pKi, namely n-hexadecanoic acid (ΔG = -9.7755 kcal/mol, pKi = 7,219) and phytol (ΔG = -7.2147 kcal/mol, pKi = 5,975) (Table 1, Fig 3).

N-hexadecanoic acid binds to cyclin D1 in glutamine, lysine and threonine. The strength of glutamine with this ligand is 25% and 2.57 Å of the distance, lysine is 63% and 2.47 Å of the distance, threonine is 62% and 2.58 Å of the distance. Phytol binds to cyclin D1 in threonine, glutamic acid, and glutamine. The strength of threonine with this ligand is 96% and 2.57 Å of the distance, glutamic acid is 27% and 1.58 Å of the distance. Glutamine is 62% and 2.58 Å of the distance (Fig 4).

Table 1. Analysis of molecular docking of ethanolic leaves extract of soursop with cyclin D1 protein

No	Compounds	ΔG (Kcal/mol)	pKi	HBond
1	3,7,11,15-Tetramethyl-2- hexadecen-1-ol	-5,9512	3,289	Gln250, Asp253
2	1,E-11,Z-13- Octadecatriene	-4,8927	3,663	-
3	7-Tetradecenal, (Z)	-5,9102	3,915	Met252
4	cis, cis, cis-7,10,13- Hexadecatrienal	-6,0333	4,023	Met252
5	2-Pentadecanol	-5,5910	3,655	Asn251
6	Oleyl Alcohol	-6,1321	3,692	Asp253
7	1,2-Benzenedicarboxylic acid, butyl octyl ester	-5,4549	3,159	-
8	**n-Hexadecanoic acid**	**-9,7755**	**7,219**	**Lys44, Thr48, Gln247**
9	Hexadecanoic acid, ethyl ester	-6,1965	4,199	Met252
10	**Phytol**	**-7,2147**	**5,975**	**Thr48, Glu60, Gln247**
11	1,2-Benzenedicarboxylic acid, diisooctyl ester	-6,4074	5,322	Thr48
12	9,12-Octadecadienoic acid, ethyl ester	-4,9061	3,635	-

ΔG = Gibbs free energy (kcal/mol); pKi = affinity; glutamine (Gln), aspartic acid (Asp), methionine (Met), asparagin (Asn), lysine (Lys), threonine (Thr), glutamate acid (Glu). The number which is next to amino acid shows amino acid position of cyclin D1 sequences.

Figure 3. 2D-ligand structure with the lowest value of ΔG and the strongest value of pKi. N-hexadecanoic acid (left), Phytol (right).

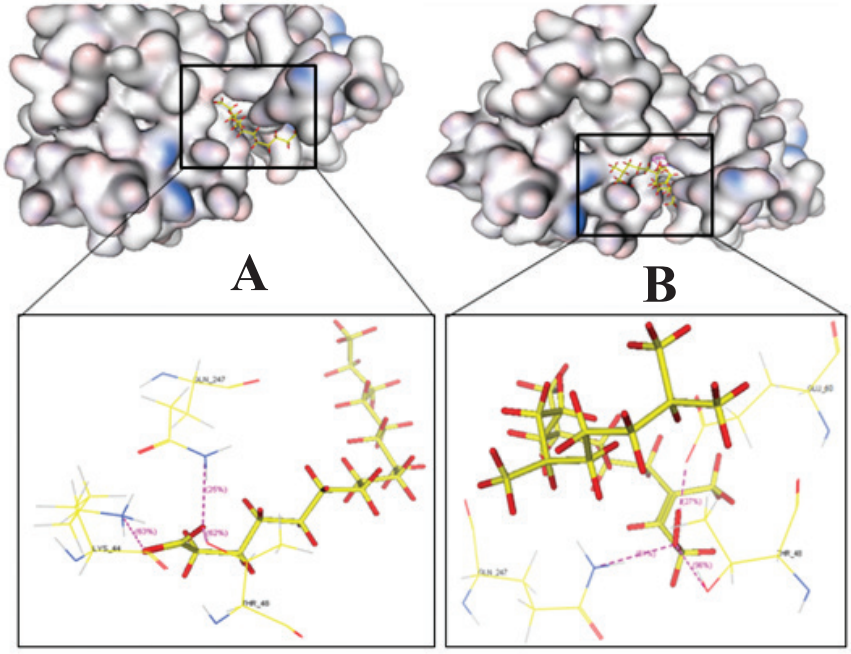

Figure 4. Molecular docking of n-hexadecanoic acid and phytol with moe software. (A) N-hexadecanoic acid binds to Gln247 (25%), Lys44 (63%), and Thr 48 (62%). (B) Phytol binds to Thr48 (96%), Glu52 (27%), and Gln247 (61%). The stick is ligand and the line is cyclin D1 protein. Yellow is carbon (C), double red line of the ligand is hydrogen (H), double red stick of protein is oxygen (O), gray is oxygen (H), and blue is nitrogen (N).

Previous study showed that soursop leaves extract can produce cytotoxic effects on colorectal cancer cell cultures such as HT-29, HCT-116,[5] COLO-205,[8] and DLD-1.[9] Soursop leaves extract is also known to reduce the expression of cyclin D1 protein in phase G1/S.[5,10] Another study showed that ethyl acetate leaves extract of soursop can induce apoptosis in rat azoxymethane-induced colonic aberrant crypt foci in rats and HT-29 cells.[18] In this study, a lower CC_{50} value indicates that the compound has greater citotoxicity activity.[19] The CC_{50} value of 5-FU is lower than the ethanolic leaves extract of soursop because 5-FU is the gold standard therapy for colorectal cancer despite combination therapy is better.[20]

The optimal concentration of ethanolic leaves extract of soursop against colorectal cancer culture cells was 148 µg/mL[9] and the optimal concentration of 5-FU against colorectal cancer culture cells was 37.4 µg/mL. The effective concentration of 5-FU that can inhibit half the population of cancer cells is 30-120 µg/mL,[21] comparable to our study that 5-FU is more cytotoxic in colorectal cancer culture cells, especially in HT-29 cells with appropriate incubation time and concentration.

Our study showed that cell viability increased with 5-FU at concentrations above 100 µg/mL whereas previous study showed 5-FU concentrations above 100 µg/mL (770 µm) were non-cytotoxic for colorectal cancer cell due to a decrease in incorporation of 5-FU.[22] Other study showed that 5-FU had limitations for incorporation with DNA or RNA, namely at a concentration of 127 pmol/ µg DNA and 1,0 pmol/ µg RNA, and causing that at such high concentrations, 5-FU could not incorporate with DNA.[23]

N-hexadecanoic acid binds to lysine44, threonine48, and glutamine247. The phytol binds to threonine 48, glutamic acid 60 and glutamine 247. The amino acid position of 31 ... 153 (44, 48, 60) is N-terminal region

of cyclin D1 protein, while the amino acid position of 156 ... 269 (247) is C-terminal region of cyclin protein D1.[24] N-terminal domain is known as cyclin box (56-145). Cyclin box is a domain that regulates binding with cyclin dependent kinase (CDK) and CDK-inhibitors.[25] Cyclin box which is inhibited by other molecules, may cause the cell cannot go to the next phase of the cell cycle. Phytol binds to the cyclin box domain, while n-hexadecanoic acid does not. Phytol that causes complex of cyclin D1-CDK4/6 cannot be formed. N-hecadecanoic acid and phytol bind to glutamine 247, that is a C-terminal region called the PEST motif (241-290). The phosphorylation of cyclin D1 degradation is threonine 286 in the PEST motif (Fig 5).[26] In this study, N-hecadecanoic acid and phytol can trigger cyclin D1 degradation.

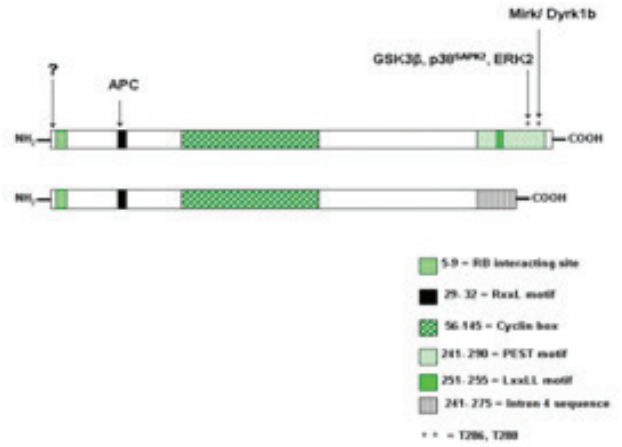

Figure 5. Cyclin D1 protein domain[26]

Molecular docking analysis with MOE obtained two molecules that have the highest ΔG and pKi, namely n-hexadecanoic acid (ΔG = -9.7755 kcal/mol, pKi = 7,219) and phytol (ΔG = -7.2147 kcal/mol, pKi = 5,975) are good categories (<-6,9 kcal/mol)because the bonds between of them are more stable. The bond only needs a little of energy and the affinity is stronger.[27]

There are three classifications of root mean square deviation (RMSD), i.e good category (RMSD ≤ 2.0 Å), category can be accepted (RMSD is between 2.0 and 3.0 Å), and bad category (RMSD≥ 3.0 Å). RMSD is a parameter used to evaluate the similarity of two structures based on the distance between two structures. The stronger bond is the closest distance between them.[28] In this study, the best RMSD is glutamic acid (1.58 Å) in phytol because it has RMSD ≤ 2.0 Å and other RMSD of amino acids are acceptable because they have RMSD between 2.0 and 3.0 Å. The two ligands have a strong bond with cyclin D1 protein, n-hexadecanoic acid can be potential as a CDK inhibitor and cyclin D1 inhibition.

In conclusion, the CC_{50} of the soursop leaves extract is higher than 5-FU and cell viability with a 2 x CC_{50} concentration of ethanolic leaves extract of soursop is lower than from 5-FU. Molecules (phytochemical components) contained in the ethanolic leaves extract of soursop inhibit the active side of the cyclin D1 protein as shown by molecular docking.

Acknowledgment

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Challenges and social support provisions in the treatment of HIV infected children in Indonesia

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Abstrak

Latar belakang: *Pengobatan pada anak yang terinfeksi HIV merupakan tantangan bagi para orang tua/ pengasuh karena berbagai permasalahan menyangkut kesehatan mereka.*

Metode: *Penelitian untuk mengeksplorasi pengalaman dan dukungan sosial pada pengobatan anak terinfeksi HIV dilakukan di 5 provinsi di Indonesia dengan prevalensi HIV tertinggi. Total sampel anak sebanyak 239 orang dari sejumlah 267 orang yang direncanakan. Data dikumpulkan dengan wawancara semi terstruktur dengan orang tua/pengasuhnya. Analisis dilakukan terhadap 165 anak berusia 1-14 tahun yang telah mendapatkan terapi obat antiretroviral.*

Hasil: *Dari sejumlah 165 anak, sebanyak 63,6% anak mengkonsumsi 1-2 item obat dan 36,4% mengkonsumsi 3-5 item. Efek samping yang paling sering terjadi adalah kulit kemerahan, mual dan muntah. Kesulitan yang paling sering dihadapi adalah rasa bosan dan anak mempertanyakan minum obat pada kelompok anak yang berusia 5-14 tahun. Orangtua/pengasuh berusaha melanjutkan pengobatan dengan mengingatkan jadwal minum obat, membujuk, memberikan penjelasan bahkan memaksa atau mengancam mereka untuk minum obat. Kesulitan tersebut makin bertambah seiring meningkatnya usia anak. Dukungan yang paling sering berasal dari orang tua dan keluarga besar seperti nenek atau paman, khususnya untuk anak yang sudah yatim.*

Kesimpulan: *Pemahaman hambatan pengobatan pada anak terinfeksi HIV dapat membantu untuk menyediakan intervensi yang tepat untuk meningkatkan kepatuhan yang akan mendorong kesuksesan terapi mereka.* ***(Health Science Journal of Indonesia 2019;10(2):103-10)***

Kata kunci: *anak terinfeksi HIV, antiretroviral, dukungan sosial, pengobatan, kesulitan*

Abstract

Background: The treatment of HIV infected children is a challenge to their caregiver due to many existing problems related to their health.

Methods: A research to explore the experience and social support on the treatment of HIV infected children was conducted in 5 provinces in Indonesia with highest prevalence of HIV. Total children sample was 239 out of previous 267 planned. Data was collected through semi structured interviews with caregivers of the children. The analysis was conducted to 165 children aged 1-14 years old who were on antiretroviral therapy.

Results: Among those 165 children, 63.6% took 1-2 items of medicines and 36.4% took 3-5 items. The most frequent adverse events were skin rash followed by nausea and vomiting. Boredom and questioning were the most frequent difficulties experienced by children aged 5-14 years old. The caregivers attempted to continue the treatment by reminding the children on schedule to take medicines, wheedling, explaining, forcing or even threatening them. The difficulties appeared more as the children grew older. The most frequent supports mainly came from parents, and extended family such as grandmother or uncle especially for orphaned children.

Conclusion: Understanding obstacles in HIV infected children will help to do proper interventions to improve adherence that will lead to successful therapy. ***(Health Science Journal of Indonesia 2019;10(2):103-10)***

Keywords: HIV infected children, antiretroviral, social support, treatment, difficulties

The ministry of Health predicted the escalation of Human Immunodeficiency Virus- Acquired Immune Deficiency Syndrome *(*HIV-AIDS) infection in children due to the escalation of new HIV infection in women, especially housewives. The number of housewives infected up to April 2017 was 12,302 that reached the highest proportion among other types of occupations. According to the report per April 2017, the number of HIV infection during 1987 to April 2017 in <1 year was 307 (0.4%), age 1-4 years old was 1,650 (1.9%), age 5-14 years old was 1,042 (1.2%) and age 15 – 19 years was 2,355 (2.7%). It was predicted that 76.7% of children age ≤ 14 years receiving Antiretroviral (ARV) of those who were eligible for therapy. The prevention mother to child transmission program (PMTCT) has been established, but the results were not yet optimal. Addressing social problems of stigma, discrimination, misconceptions and male involvement must be the part of PMTCT package.[1-3]

HIV-AIDS treatment is a lifelong treatment so that the continuity of treatment affects the outcome of treatment. The aims of antiretroviral (ARV) treatment are to reduce morbidity and mortality associated with HIV, improve the quality of life of people living with HIV-AIDS, restoring and maintaining immune function, maximizing virus replication suppression as long as possible.[4] HIV infected children are more vulnerable to treatment access because they have not been independent and often become a burden on family or relatives because their parents have died of HIV-AIDS.

Child Protection Act stated that every child has the right to live, grow, develop and participate fairly in accordance with human dignity, as well as protection from violence and discrimination. Countries and governments are obliged and responsible for providing infrastructure support in the implementation of child protection. HIV infected children and stigmatized children caused by their parents' status are stated in article no 59 as those who are eligible to have special type of protection by conducting appropriate monitoring, prevention, cure, treatment and rehabilitation program.[5] Thus, HIV infected children treatment program is considered as a form of children protection.

Studies conducted by UNICEF and the National AIDS Commission indicate the difficulties faced by children infected with HIV-AIDS to access education and health services due to discrimination, family's financial hardship, worsen child health and the need to care for the elderly who were also HIV-AIDS positive.[3] Lack of assistance on nutrition, monitoring and forgetfulness to take ARV caused poor adherence.[6] The caregivers of HIV positive children in poor conditions showed a high level of stress.[7] Approximately 90% of about 3.4 million HIV infected children lived in sub-Saharan Africa, and more than 500 thousand were infected through perinatal process. The implication is on the need to have adequate care and treatment. Since the use of antiretroviral treatment (ART), those children with access to ARV can live longer and healthier lives into adolescence and adulthood. Therefore, pediatric HIV care programs should shift its focus from survival to addressing children's physical and psychosocial wellbeing. The presence of parents who take care of the children, involving psychosocial interventions during treatment and expanding family support center indicated as the factor for successful treatment.[8-10] This study aims to explore the challenges faced by children and their caregiver during ARV therapy and the efforts of caregiver to support adherence.

METHODS

Data sites

Based on the report, the sites of data collection were in five provinces with high prevalence of HIV in Indonesia with consideration of west to east areas. The three monthly reports of Directorate general of Disease Prevention and Control in 2013 stated that the number of HIV-AIDS cases mostly located in DKI Jakarta, East Java, Papua, West Java, Bali, North Sumatera, Central Java, West Borneo, Riau Island and South Sulawesi provinces. We selected five provinces with highest number of cases except for West Java as it is located close to DKI Jakarta which has been chosen. In each province was chosen as a city and a district. The provinces were DKI Jakarta (West Jakarta and North Jakarta), East Java (Surabaya and Malang), Bali (Denpasar and Buleleng), North Sumatera (Medan and Deli Serdang) and Papua (Jayapura city and Jayapura district) as listed in table 1. Data was collected from March-July 2015. The data was part of a research entitled Akses Pengobatan HIV-AIDS dan Infeksi Oportunistik pada Anak di Sepuluh Kabupaten/Kota di Indonesia (Access to HIV-AIDS and Opportunistic Infection Treatment of Children in Ten Districts in Indonesia).

Data collection

Based on the classification that children are those age less or similar to 19 years old, we considered

prioritizing the guardians/caregivers as the main informants. The sample was the guardians/caregivers of children with HIV-AIDS aged less or similar to 19 years old. Children aged 15 or more with ability to answer by themselves were also eligible for sample informants. Therefore, the respondent can be the caregivers or the children themselves depend on the age of children. The number of caregiver of children to be interviewed was based on the number of children with HIV-AIDS as reported in the district health offices in ten districts sample areas, regardless the possibility of caregivers with more than one HIV positive children. The guardians were interviewed personally by the researcher using semi structured interview form. In addition to those caregivers, relevant health care providers and nongovernmental organizations (NGO) staff were also been interviewed using in depth interview form.

The number of guardians/caregivers was counted based on the number of reported HIV cases in children using the formula[11]:

$$n = \frac{Z^2\ P(1-P)}{(d)^2}$$

n = sample size Z = 1.96 P = 50 % d = 0.06

The number of samples was 267 respondents. The number of samples in each district decided using proportional sampling method.

$$n1 = \frac{n.\ N}{N1}$$

n1 = proposed sample in district 1; n = sample number in district 1 ; N = number of total sample (267); N1 = total population (517, based on national report)

Table 1. Number of sample reported, proposed and interviewed (0-19 years old)

NO	Province	District	N reported	N proposed	N sample	% response rate
1	DKI Jakarta	North Jakarta	71	39	45	115,38
		West Jakarta	35	17	21	123,53
2	East Java	Surabaya City	87	46	44	95,65
		Malang District	36	17	16	94,12
3	Bali	Denpasar City	51	28	24	85,71
		Buleleng District	16	9	19	211,11
4	Papua	Jayapura City	93	45	22	48,89
		Jayapura District	44	25	15	60,00
5	North Sumatera	Medan City	76	35	22	62,86
		Deli Serdang District	8	6	11	183,33
	Total guardians/caregivers		517	267	239	89,51

Data analysis

Due to consideration that the range of age with vulnerability of transmission from mother was 1-14 years old which was also the category of age used by ministry of Health in its report, we only analyzed caregivers who have children 1-14 years old. The problems of treatment mostly happen in those who were on antiretroviral (ARV) therapy. Therefore, a further analysis was conducted for those aged 1-14 years old and also on ARV. As a result, those aged less than 1 year or more than 14 years (who were interviewed directly) were excluded in the analysis.

The variables to be analyzed were characteristics of children including age groups and number of drugs taken; caregivers' status including education, occupation, and relationship with children. Variables related to ARV treatment were viewed on two sides: adverse events and challenges impeding treatment, and social support to continue treatment. Both inhibiting and supporting factors were analyzed using quantitative and qualitative methods. We collapsed the characteristic, adverse events, challenges, and social support form and sources into frequency distribution table which also completed with qualitative analysis based on verbal narration of the caregivers. Quantitative data were analyzed using SPSS v.18 to present the frequency distribution characteristic and bivariate analysis on the relationship between children and caregiver characteristics with compliance of period to refill the medication; the relationship of children characteristics with adverse events and impediments; and also the relationship of caregiver status with supporting efforts. Qualitative data resulted from in depth interviews with the health care providers and NGO staff were analyzed descriptively as an addition to quantitative data.

Ethical approval

Ethical approval was obtained from the Institutional Review Board in National Institute of Health Research and Development, number LB.02.01/5.2/KE.071/2014.

RESULTS

Characteristic of children

The number of children aged 1-14 years old who were on ARV was 165 among all 239 samples. Most caregivers interviewed were the biological parents, mainly the mothers. The site of living, characteristics of the children, number of drugs items taken, caregivers' education status, house status and relationship with children are listed in table 2.

Table 2. Characteristic of children and caregivers

Characteristic	N	%
District		
North Jakarta	35	21.2
West Jakarta	21	12.7
Surabaya City	30	18.2
Malang District	6	3.6
Denpasar City	21	12.7
Buleleng District	16	9.7
Jayapura City	4	2.4
Jayapura District	3	1.8
Medan City	22	13.3
Deli Serdang District	7	4.2
Age range		
1-4 years	46	27.9
5-14 years	119	72.1
Number of drugs taken		
1-2 items	105	63.6
> 2 items	60	36.4
Education of caregivers		
Up to junior high school	82	49.7
Senior high school and up	83	50.3
Occupation of caregivers		
Housewives/unemployed	80	48.5
Employed	85	51.5
Relationship with children		
Biological parents	109	66.1
Extended family or others	56	33.9
Compliance on routine medication refill		
At least every month	161	97.6
More than one month/irregular	4	2.4
Total	**165**	**100**

Compliance on routine medication refill

Bivariate analysis is conducted in order to find out the relationship between characteristic and compliance. The limitation of this research did not directly measure compliance on taking medication in detail by counting the leftover medication or asking how many times they did not take ARV. The standard period of refill medication regularly is usually one month. Therefore, those who went to health facilities less than once a month are considered as having the possibility of incompliance. Yet the proportion of children who were considered comply in refilling the medicine was 97.4%. This proportion is too high to find a correlation between those who complied and those who did not. The analysis showed in table 3.

Table 3. The relationship between characteristic and compliance on routine medication refill

Characteristic	Comply on routine medication refill		Exact Sig (2 sided)
	n	%	
Characteristic of children			
Age range			
1-4 years	44	95.7	0.310
5-14 years	117	98.3	
Number of drugs taken			
1-2 items	102	97.1	1.000
> 2 items	59	98.3	
Characteristic of caregivers			
Education			
Up to junior high school	81	98.8	0.620
Senior high school and up	80	96.4	
Occupation			
Housewives/not working	78	97.5	1.000
Working	83	97.6	
Relationship with children			
Biological parents	105	96.3	0.301
Extended family or others	56	100.0	

As predicted, the result shows that there is no significant factor of compliance to refill the medication, neither characteristic of children nor characteristic of caregivers.

Challenges

In addition to a number of drugs that should be consumed by children as listed in table 2, the adverse event was also one of the barriers to regular treatment. Experienced adverse events can be more than one type. Reported adverse events and faced challenges are listed in table 4.

Despair was experienced by some children and parents. Parents desperate when they should give bitter medicine in children under 5 years due to the unavailability of pediatric dosage form. The desperation could possibly lead a low level of adherence to stop taking medication.

> "[When they] Got a problem, then despair, the parents are deceased, for what life is, what to do anyway [as] I have an illness like this, I shall pass [away]." (NGO, Surabaya)

The parents' discipline to take the drug from hospitals was still lacking and many patients who went half-heartedly so often end up in drop out, especially when experiencing side effects.

> "The HIV positive parents are lazy to take medicines, so the children do not take it also. They said they surrender, alive or dead is up to God." (NGO, Kab. Malang)

Table 4. Challenges in children's treatment

Aspects	Number of drugs taken					Age range				
	1-2 items		> 2 items		Exact Sig (2 sided)	1-4 years		5-14 years		Exact Sig (2 sided)
	n	%	n	%		n	%	n	%	
Adverse event										
Nausea/vomiting	14	13.3	12	20.0	0.274	7	15.2	19	16.0	1.000
Dizziness	10	9.5	4	6.7	0.772	1	2.2	13	10.9	0.115
Fatigues/drowsiness	13	12.4	8	13.3	1.000	3	6.5	18	15.1	0.193
Insomnia	14	13.3	4	6.7	0.299	4	8.7	14	11.8	0.782
Skin rash	27	25.7	18	30.0	0.558	10	21.7	35	29.4	0,436
Anemia	3	2.9	6	10.0	0.074	1	2.2	8	6.7	0,447
Challenges										
Boring	40	38.1	21	35.0	0.739	11	23.9	50	42.0	**0.033***
Forgetfulness	9	8.6	8	13.3	0.425	1	2.2	16	13.4	**0.043***
Hard to swallow / vomiting	9	8.6	11	18.3	0.083	9	19.6	11	9.2	0.107
Keep questioning	40	38.1	21	35.0	0.739	8	17.4	53	44.5	**0.001***

Table 4 shows the relationship of number of drugs taken and age range of children with adverse events and challenges (impediments) happened. It can be concluded that number of drugs taken has no significant influence on adverse events and challenges. Contrarily, age range shows several significant influences challenges aspect. Older children faced more adverse events and challenges in taking ARV. Older children can express their feeling better than younger children. The same assumption might also for boredom and forgetfulness, in which older children experienced them more frequently. Meanwhile keep questioning on why they have to keep taking medication possibly happened to older children as they can ask. The older the age of children the more they think about why they must take the medication while others don't. Younger children can be forced to take medication. Even though it is not significance but the problem of hard to swallow or vomiting happened more frequently in younger children due to bitterness or tablet dosage form.

Social Supports

Support to sustain regular treatment was done in various ways. The support can be more than one way and the source of support can be derived from more than one source. The caregivers admitted various sources of support which comes mostly from family (75.2%), NGOs/peer groups (51.5%), caregivers' spouse (47.9%), and healthcare providers (44.8%). The relationship of caregivers characteristic and the form of social support are listed in table 5.

Some children do not experience difficulty in taking medications, especially babies because they could not refuse. However some children must be persuaded or even forced by 3 adults in a case. Most parents try to give medication regularly and some HIV-positive parents gave their own example by taking medicine to induce the child to imitate them. Social support came mostly from family although initially one or more family members deny or discriminate against his/her own HIV positive family.

> "Initially there was shocked, but slowly they will think again who else if not me that support. If you already know, most of the support and compassion came from them." (NGO, Jakarta Barat)

> "Children are the nation's capital, so they must be handled properly" (hospital physician, Kota Denpasar)

> "K is the same with her father; she exactly knows that she doesn't want to be like her father as her father has passed away." (NGO, Kab. Buleleng)

> "Just don't ever, ever there will be a story that there were curly black haired people in Papua". (NGO Kab. Jayapura)

The last quote implies a very deep concern and worries about the future existence of Papua people. If HIV-AIDS continues to spread out in general population, more and more people die due to AIDS, then in the future Papua people will be left as history.

Various forms of social support already existed in the form of support from government agencies or NGOs. Various NGOs have been exist in each district / city with specialty in handling HIV children, certain groups of people living with HIV and people living with HIV in general. In Buleleng NGO volunteers took ARV to health centres or referral hospitals. Some volunteers and health workers also provided home visits to monitor the treatment of patients.

Social support from the community, among others were the existence of groups such as AIDS care community in East Java and village cadres concerned with AIDS community and students care with AIDS and Drugs community in Bali. In Papua there are traditional leaders and religious leaders who have been involved in HIV-AIDS. One of the advantages in Papua is their chiefs (Ondo Api) who were very supportive by approaching the traditional leaders first.

Table 5. The forms of supporting efforts

Characteristic of caregivers	**Category 1**		**Category 2**		
Working status	**Housewives/ unemployed**		**Working/ Employed**		**Exact Sig (2 sided)**
	n	**%**	**n**	**%**	
The form of support					
Reminding	44	55.0	51	60.0	0.516
Persuading	39	48.8	47	55.3	0.400
Forcing/making scared of	14	17.5	9	10.6	0.200
Explaining	20	25.0	32	37.6	0.081
Education	**Up to junior high school**		**Senior high school and up**		**Exact Sig (2 sided)**
The form of support	**n**	**%**	**n**	**%**	
Reminding	52	63.4	43	51.8	0.694
Persuading	44	53.7	42	50.6	0.694
Forcing/making scared of	11	13.4	12	14.5	0.847
Explaining	24	29.3	28	33.7	0.532
Relationship	**Biological parents**		**Extended family or others**		**Exact Sig (2 sided)**
The form of support	**n**	**%**	**n**	**%**	
Reminding	59	54.1	36	64.3	0.211
Persuading	61	56.0	25	44.6	0.168
Forcing/making scared of	14	12.8	9	16.1	0.571
Explaining	36	33.0	16	28.6	0.560

Table 5 explored the relationship of caregivers' characteristics and the form of efforts to support compliance on taking medication. Unfortunately there is no significant difference in types of efforts between caregivers with different characteristics.

DISCUSSIONS

Antiretroviral therapy for people living with HIV AIDS (PLHIV) is aimed to prolong the life of PLHIV as well as their quality of life. Due to lifelong treatment, adherence in the therapy process becomes a critical point of successful treatment.[12] Antiretroviral therapy in children also faces an adherence problem in regard to similar or different factors. In Ethiopia about 60% of eligible PLHIV have been treated by antiretroviral but only 12% of children aged less than 15 years getting it because of data limitations and lack of awareness about the possibility of infection in children especially those who are older.[13] This result showed similarity of problems in taking medication in older children even though they still comply with the therapy. There is no significant factor of adherence in term of number of pills, education background and occupation of caregivers and even the relationship of caregivers. This result is in accordance with previous research which showed that the predictors were not significantly influence adherence except the unavailability of caregivers.[10]

Children are considered as a vulnerable subject in which the success of the treatment highly depends on their caregivers. Estimated number of orphans because

their parents died due to AIDS in Ethiopia was about 800,000. Approximately 11.9% of the children infected with HIV-AIDS and mostly aged between 5-10 years were from infected parents but were not identified and does not have access to treatment.[13] A key factor in compliance with ARV treatment in pediatric patients is the presence of biological parents who care for children.[10,14] More PLHIV can be saved since the development of antiretroviral, so that fewer and fewer children are being orphaned and otherwise the children were raised by parents with the status of PLHIV even though some may be asymptomatic.[15]

Children and teenagers infected HIV from their parents is a challenge in HIV prevention because there is no comprehensive intervention concerning the problems of puberty, psychosocial and neurocognitive function.[16] Many problems related to children's adherence have been identified from their caregivers ranging from an economic problems, stressful, forgetful, older caregivers, growing older children and ARV related problems.[6, 7, 10, 17-20] ARV related problems might due to adverse events such as skin rash, nausea/vomiting and dizziness. Our result showed similar problem with previous research which mainly stated that boredom, forgetfulness and questioning children were the most common problems as the children growing older. The older the children are, the more questioning they are.

Sustaining the treatment becomes a challenge for caregivers. This study also highlights the efforts made by caregivers in the form of reminding schedule, persuading and sometimes attributed by forcing or threatening children to take medicines although there is no significant background factor from different approaches. Previous research also revealed the same attempts made, even in elderly caregivers.[20] The need for social support for caregivers and the children themselves is clearly shown which can be started from disclosing status and counselling program.[21,22] Decreasing a burden through disclosing status proved to increase the success of treatment.[8,23] In addition, our study also emphasized that the main source of support in children receiving ARV came from family either the biological parents or grandparents and extended family.

To conclude, maintaining adherence in children needs policies and programs addressing the problems shown. Interventions should include elements to improve the welfare of the school to reduce stigma and create a more supportive environment such as family support centres.[9] Providing guidelines for disclosing status and counselling are also needed to support the caregivers.[10,24,25] Innovative interventions should enjoin education, counselling, provision of social support, directly observed therapy, and financial incentives. Some successful methods included reminder using text messaging and pillboxes.[12] In this case, parents can be equipped with pillboxes and also supported by NGO or health provider staff by using text messaging service or other feasible communication media.

Understanding the problem related to treatment and exploring the available social support is needed to identify the possible solutions. This research provides needed information about challenges and social supports. Difficulties on HIV infected children treatment mainly due to boredom and children questioning on long time medication. Efforts made by the caregivers by reminding or persuading the children to take medicines, yet support from family emerged as an important form of social support.

In conclusion, the treatment of HIV positive children is challenged by many problems such as the problem related to antiretroviral, psychosocial problem and other problems from caregivers. Boredom, forgetfulness and keep questioning on why they have to take the medication revealed as significant challenges in the treatment of older children (aged 5-14 years old). Successful treatment cannot be achieved unless relevant interventions involving all related sectors including government, health provider, NGOs and school are made for supporting the children and their families.

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Perceptions of pregnant woman on monetary and time sacrifice for satisfaction based on health workers roles in antenatal services to reduce the risk of maternal death at Gowa district

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Abstrak

Latar belakang: *Angka Kematian Ibu (AKI) merupakan salah satu indicator pembangunan kesehatan di Indonesia. Upaya percepatan penurunan AKI dapat dilakukan dengan menjamin agar setiap ibu mampu mengakses pelayanan kesehatan ibu hamil yang berkualitas. Apabila antenatal care dimanfaatkan dengan baik maka kesehatan ibu dapat terpantau secara berkesinambungan dari masa kehamilan sampai dengan persalinan.*

Metode: *Desain penelitian adalah observasional dengan rancangan cross sectional study. Populasi pada penelitian ini adalah semua ibu hamil yang memiliki usia kehamilan 7-9 bulan di Kabupaten Gowa sebanyak 122 orang. Sampel sebanyak 93 orang diambil dengan menggunakan teknik accidental sampling.*

Hasil: *Ibu hamil yang memiliki persepsi pengorbanan moneter kecil dan mengatakan peran petugas kesehatan kurang dalam pelayanan antenatal sebanyak 90,0%. Ibu hamil yang memiliki persepsi pengorbanan moneter sangat kecildan mengatakan peran petugas kesehatan kurang sebanyak 83.1%. Ibu hamil yang memiliki persepsi pengorbanan waktu besar dan mengatakan peran petugas kesehatan kurang dalam pelayanan antenatal sebanyak 100%. Ibu hamil yang memiliki persepsi pengorbanan moneter besardan mengatakan peran petugas kesehatan kurang dalam pelayanan antenatal sebanyak 90.2%.*

Kesimpulan: *Kepuasan ibu hamil terhadap peran petugas kesehatan dalam pelayanan antenatal berdasarkan persepsi pengorbanan moneter tidak menunjukkan korelasi sedangkan berdasarkan pengorbanan waktu menunjukkan ada korelasi. Perlu meningkatkan kecepatan proses pelayanan pemeriksaan kehamilan pada ibu hamil oleh tenaga kesehatan.* ***(Health Science Journal of Indonesia 2019;10(2):111-8)***

Kata kunci: *Pelayanan antenatal, Ibu Hamil, Pengorbanan, Waktu, Moneter*

Abstract

Background: The Maternal Mortality Rate (MMR) is one indicator of health development in Indonesia. Efforts to accelerate the reduction of MMR can be done by ensuring that every mother can access quality maternal health services. Antenatal care is utilized properly, maternal health can be monitored continuously from pregnancy to delivery.

Methods: The study design was observational with a cross sectional study design. The population in this study were all pregnant women who had a gestational age of 7-9 months in Gowa Regency as many as 122 people. A sample of 93 people was taken using accidental sampling technique.

Results: Pregnant women who have a perception of small monetary sacrifice and say the role of the health workers is lacking in antenatal care 90.0%. Pregnant women who have a perception of monetary sacrifice are very small and say the role of health workers is less as much as 83.1%. Pregnant women who have the perception of the sacrifice of big time and say the role of health workers lacking in antenatal care as much as 100%. Pregnant women who have a perception great monetary sacrifice and say the role of health workers is lacking in antenatal care 90.2%.

Conclusion: Satisfaction of pregnant women towards the role health workers in antenatal care based on perception monetary sacrifice does not show correlation while based on time sacrifice shows there is a correlation. Need to increase the speed of the process of pregnancy examination services for pregnant women by health workers. ***(Health Science Journal of Indonesia 2019;10(2):111-8)***

Keywords: *Antenatal care, Pregnant Women, Sacrifice, Time, Monetary*

Every year around the world, an estimated 358,000 maternal deaths occur and around 99% of those deaths occur in poor developing countries including Indonesia.[1] Maternal death is a complex event caused by various causes that can be distinguished by determinants of close, between, and far.[2] Close determinants that are directly related to maternal death are obstetric disorders such as bleeding, preeclampsia/ eclampsia, and infections or illnesses suffered by the mother before or during pregnancy that can worsen pregnancy conditions such as heart, malaria, tuberculosis, kidney, and acquired immunodeficiency syndrome. Close determinants are directly affected by determinants between those related to health factors, such as maternal health status, reproductive status, access to health services and the behavior of using health facilities. Determinants are far related to demographic and sociocultural factors.[3]

The success of maternal health efforts can be seen from the indicators of Maternal Mortality Rate (MMR). The MMR is the number of maternal deaths during pregnancy, childbirth and childbirth caused by pregnancy, childbirth, and post partum or its management but not due to other causes such as accidents or falls in every 100,000 live births.

Efforts to accelerate the reduction of MMR can be done by ensuring that every mother can access quality maternal health services, such as health services for pregnant women, delivery assistance by trained health workers in health care facilities, postpartum care for mothers and babies, special care and referrals if there are complications, ease of getting maternity and maternity leave, and family planning services.[4,5]

The World Health Organization (WHO) recommends that the obligation to visit ANC during normal pregnancy is 4 visits during pregnancy with a predetermined standard and time. K1 was increased but K4 is decreased.According to the Indonesian Health Profile in 2015, ANC coverage in Indonesia for K1 was 95.75% and K4 coverage was 87.48% (Ministry of health, 2015). According to Indonesia's Health Profile in 2017, ANC coverage in Indonesia for K4 in 2016 was 85.35% and K4 coverage in 2017 was 87.3%.[6,7]

The coverage of K4 health services for pregnant women by Province in 2017 was 76%. However, of all the provinces in Indonesia, there are 11 provinces that have not reached the target of the strategic plan. Constraints faced in the implementation of health services for pregnant women are not only in terms of access. The quality of services provided must also be improved, including the fulfillment of all components of health services for pregnant women must be provided at the visit. In terms of availability of health facilities, up to December 2017 there were 9,825 health centers. The existence of an ideal health center must be supported by good accessibility. This of course is closely related to geographical aspects and the ease of transportation infrastructure. In supporting outreach to the community in its working area, Health Center has also applied the concept of satellite by providing auxiliary health center.[8]

The maternal deaths Mortality Rate (MMR) in South Sulawesi province year in 2016 and 2017 cases of maternal mortality were recorded as 156 and 115, this shows a decrease in cases of maternal death in 2017 as many as 41 cases. The number of mothers doing K1 ANC in Gowa Regency was 165,777 people.[7]

The maternal deaths Mortality Rate (MMR) in Gowa Regency in 2016 was recorded at 18 people, while in 2017 as many as 13 people. Percentage of K1 service coverage in 2017, K1 service coverage was 107.28% and K4 was 108.100%. In 2018, K1 service coverage was 104.32% and K4 was 97.62%.[8]

K4 visits for pregnant women are the most important visits to be done by pregnant women because at K4 visits pregnant women get complete services as they should according to the standards set. This study aims to assess the role of health workers in antenatal care based on perceptions of monetary sacrifice and the time of pregnant women to reduce the risk of maternal death.

METHODS

This is an Observational research with cross sectional type, meaning that each research subject is only observed once and the measurement between independent and dependent variables is done at the same time.

Gowa District Health Center data for 2017, from the target of 257 pregnant women for K1 coverage to 254 people (98.8%) while for K4 coverage to 244 people (95%), for 2018 with 252 pregnant women targeting for K1 coverage 257 people (102%) while K4 coverage reached 241 people (95.6%), for 2019 in February the number of pregnant women was recorded at 258. While for K1 coverage were 47 people (18.2%) and for K4 coverage was 40 people (15.5%). While the expected health center target is 100% per year.

The population in this study were all pregnant women who had a gestational age of 7 to 9 monthsin Gowa Regency during the study period of 122 people. The sample in this study were pregnant women who had a gestational age of 7 to 9 months in Gowa Regency taken using accidental sampling, which is a sampling technique based on chance.[9] Where pregnant women found at the Gowa District Health Center by chance were determined as samples. The sample size was determined using the Slovin formula of 93 people.

$$n = \frac{N}{1 + (N.e^2)}$$

Information:
n = Sample Size
N = Large Population
e = Standard Error (0.05)
So that it gets:

$$n = \frac{N}{1 + (N.e^2)}$$

$$= \frac{122}{1 + (122.0{,}05^2)}$$

$$= \frac{122}{1 + (122.0{,}0025)}$$

$$= \frac{122}{1{,}305}$$

$$= 93$$

Data were obtained directly using a questionnaire for pregnant women found at the Gowa District Health Center and were willing to participate in the study. Samples were interviewed after having ANC at the health center.

The health workers in this studied include all the people that give services to pregnant women to get antenatal care in health center like that administrative staffs, midwives, nutritionist for nutrition, nurses, public health workes for health promotion, doctor for qualified ANC in which one of the visit should be examined by the doctor.

Monetary Sacrifice is a sacrifice related to the perception of the costs incurred by pregnant women in utilizing antenatal care. Monetary perceptions, such as: Transportation Costs, Voluntary Costs, Other Costs.

Sacrifice Time is the perception of time inherent in the use of antenatal care that involves the time spent by pregnant women in all aspects of the antenatal care process. Perception of time, such as: Long time on the trip, long time on the queue, long time on service, etc.

Monetary sacrifice and time are measured on a Likert scale and Scoring is done on each item with a total number of questions of 10 (ten) questions. Each answer is given a score. Very small (4), small (3), big (2), and very large (1). Use the interval formula as follows:

Highest score = 10 x 4 = 40 (100%) and Lowest score = 10 x 1 = 10 (10 / 12x100% = 25%)
Range = highest score - lowest score
= 100% - 25% = 75%

Then, Information:
I = interval R = Range (highest score - lowest score)
K = Number of categories
"I" = R / K = "I" = 75/4 = 18.75%

The desired score is:
Highest score - Interval = 100% - 18.75% = 81.25%
Objective Criteria:
Very Small: If the score obtained by respondents 81.25 - 100%
Small: If the score obtained by respondents 62.49 - 81.24%
Large: If the score obtained by respondents is 43.73 - 62.48%
Very Large: If the score obtained by respondents <43.72

Pregnant mothers' satisfaction with antenatal care services can be measured through perceptions of pregnant women regarding the number of visits, time of visit, and recommended procedures or service components that contain 10T standards when conducting antenatal care visits.

Satisfaction Pregnant Women to The Role of Health Care Workers in Antenatal Care are measured on a Likert scale and Scoring is done on each item with a total number of questions of 10 (ten) questions. Each answer is given a score. Very Satisfied (4), Satisfied (3), Not Satisfied (2), and very dissatisfied (1). Use the Arrange as above:

Highest score = 10 x 4 = 40 (100%)
Lowest score = 10 x 1 = 3 (3 / 12x100% = 25%)
Range = highest score - lowest score
= 100% - 25%
= 75%
Then,
Information:
I = interval
R = Range (highest score - lowest score)
K = Number of categories
"I" = R / K
"I" = 75/2
= 37.5%

The desired score is:
Highest score - Interval = 100% - 37.5%
= 62.5%
Objective Criteria:
Good: If the score obtained by respondents ≥62.5%
Not Good: If the score obtained by respondents <62.5%

Analysis of the data in this study is Correlation analysis with pearson correlation test. Correlation is a tool used to measure the level of closeness of the relationship between independent variables with the dependent variable. Analysis data also use chi square test. The presentation of data is done in the form of frequency and percentage distribution tables accompanied by an explanation.

Ethical Declaration

This study has been approved by the Ethics Committee Health Research of Universitas Muslim Indonesia and Ibnu Sina YW-UMI Hospital number 095/A.1/KEPK-UMI/V/2019.

RESULTS

The results of research on the role of health workers in antenatal care based on perceptions of monetary sacrifice and time of pregnant women to reduce the risk of maternal death are shown as follows:

Table 1. Distribution of mothers based on characteristics

Characteristicts Respondents	n=93	%=100
Age (years)		
<20	1	1.1
20-35	81	87.1
>35	11	11.8
Gravid		
1	25	26.9
2	21	22.6
>3	47	50.6
Education Level		
Never attended school	4	4.3
Not graduated Elementary School	3	3.2
Elementary school	20	21.5
Middle School	36	38.7
High school	25	26.9
University	5	5.4
Family Income		
< Rp. 2.860.000	47	51,8
≥2.860.000	46	48,5
Husband occupation		
Labor	33	35,5
Fisherman	2	2,2
Private employees	7	7,5
Farmers	23	24,7
Car Driver	1	1,1
Entrepreneur	27	29,1

Source: Primary Data 2019

Table 1 showed that the majority of pregnant women are aged 20-35 years (87.1%), primigravid (26.9%) and multigravida (50.6%), the education level of the majority is middle school (38.7%), high school (26.9%), family income < 2.860.000 (51.8%), and husban jobs as labor (35.5%), farmers (24.7%), entrepreneurs (29.1%).

Table 2. Distribution of mothers based on perceptions of monetary sacrifice in antenatal services in Gowa Regency in 2019

Monetary Sacrifice Perception	n=93	%=100
Sacrifice in Transportation Costs		
Very Large	0	0
Big	12	12.9
Small	53	57.0
Very Small	28	30.1
Service Administration Cost Sacrifice		
Very Large	0	0
Big	2	2.0
Small	23	24.7
Very Small	68	73.1
Photocopy Cost Sacrifice		
Very Large	0	0
Big	0	0
Small	8	8.6
Very Small	85	91.4

Source: Primary Data 2019

Table 2 showed that the majority of perceptions of pregnant women regarding the sacrifice of transportation costs fall into the small category (57%), the sacrifice of administrative services costs is very small (73.1%), and the sacrifice of photocopy costs falls into the very small category (91.4%).

Table 3. Distribution of mothers based on perceptions of time sacrifice in antenatal services in Gowa Regency in 2019

Perception of Time Sacrifice	n=93	%=100
Time / Travel LengthSacrifice		
Very Large	2	2.2
Big	11	11.8
Small	53	57.0
Very Small	27	29.0
Long Time Queue Sacrifice		
Very Large	4	4.3
Big	55	59.1
Small	20	21.5
Very Small	14	15.1
Long Service Time Sacrifice		
Very Large	2	2.2
Big	42	45.2
Small	34	36.6
Very Small	15	16.1

Source: Primary Data 2019

Table 4. The role of health care workers in antenatal services based on monetary sacrifice perception and time of pregnant women as an effort to reduce the risk of maternal death

Mother Sacrifices	Satisfaction Pregnant Women to The Role of Health Care Workers in Antenatal Care				Total		Nilai p
	Good		Not good				
	n	%	n	%	n=93	%=100	
Monetary Sacrifice							p=0.582* r=0.058**
Very Large	0	0	0	0	0	0	
Big	0	0	0	0	0	0	
Small	9	90,0	1	10,0	10	100	
Very Small	69	83,1	14	16,9	83	100	
Time Sacrifice							p=0.09* r=0.930**
Very Large	0	0,0	2	100	2	100	
Big	4	9,8	37	90,2	41	100	
Small	25	75,8	8	24,2	33	100	
Very Small	14	82,4	3	17,6	17	100	

Source: Primary Data 2019

*Uji Chi Square

**Uji Korelasi Pearson

Table 3 shows that the majority of perceptions of pregnant women regarding the sacrifice of Time/ Travel Lengthinto the small category (57%), the sacrifice of Long Time Queueis large (59.1%), and the sacrifice of long service time into the large category (45.2%).

Table 4 shows that maternal satisfaction with the role of officers in antenatal care does not correlate with the perception of monetary sacrifice. On the other hand the satisfaction of pregnant women towards the role of officers in antenatal care correlates with the perception of sacrifice of time.

DISCUSSIONS

Based on the results of the research and data processing that has been presented, this discussion will explain in accordance with the research objectives, namely: how the role of health workers in antenatal care based on the perception of monetary sacrifice and time of pregnant women as an effort to reduce the risk of maternal death.

1. The role of health workers in antenatal care as an effort to reduce the risk of maternal death

Pregnancy Checkup or Antenatal Care is an activity given to the mother before giving birth or during pregnancy. Pregnancy examination is an effort made in the maintenance of the health of the mother and her womb. Pregnancy checks should be done at least four times during pregnancy, namely once in the first trimester, once in the second trimester, and twice in the third trimester. Pregnancy examination is needed because although in general the pregnancy develops normally and results in healthy births of the baby through the birth canal, sometimes it is not as expected.[10,11,12,13,14]

Antenatal Care is a planned program in the form of observation, education, and treatment of medicines for pregnant women, with the aim to keep the mother healthy during pregnancy, childbirth and childbirth as well as maintain a healthy born baby, the process of pregnancy and childbirth that is safe and satisfying, monitoring the possible risks of pregnancy, and reduce maternal and fetal perinatal morbidity and mortality.[15,16]

The results showed that the majority of respondents at 83.9% rated the role of health workers in antenatal services as good. The use of antenatal care (ANC) in health center is still low or underutilized. From the results of the study most of the respondents said they did not use the pregnancy check up at the health center. They preferred to have their pregnancy checked by the village midwife, because the distance to the village midwife is closer than to the health center. Besides, at the village midwife the pregnancy check-up process did not take so long, and there was no queue.

The results showed that the service that was not good according to pregnant women was that the waiting time to obtain antenatal services was very long,

whether it was in the card room for administrative processes, the service room of midwives / doctors / other health workers, and in the medical waiting room. So that pregnant women feel a great sacrifice of time in obtaining antenatal care. So it needs efforts to improve service quality in terms of service time. The quality of ANC services based on the 10T standard is not optimal, there are still some services provided that are not in accordance with the 10T standard. The administrative process for registration is very complicated and complicated.

The results showed that the service that was good according to pregnant women was that the health staff's friendliness, facilities and infrastructure were quite adequate, the quality of the ANC was already good from the aspect of providing information about the ANC, the provision of the MCH handbook, the implementation of some good ANC standards.

2. The satisfaction of pregnant woman to the role of health workers in antenatal care based on perceptions of maternal monetary sacrifice in an effort to reduce the risk of maternal death

Monetary sacrifice is a cost incurred by the customer to obtain goods or services that greatly affect customer satisfaction in utilizing the goods or services.Monetary sacrifice is the sacrifice of money that must be paid by customers to obtain products or services that will be used Kotler & Keller, 2009. Monetary sacrifice is related to the financial costs incurred by the customer in utilizing antenatal care.[17,18]

The results of research on the relationship between satisfaction of pregnant women to the role of health workers in antenatal care based on the perception of maternal monetary sacrifice show the results of correlation analysis of 0.058 (p = 0.582) which means no correlation. Results of research on evaluating the perception of monetary sacrifice based on the role of health workers in antenatal care show that at a small monetary sacrifice of 90% stated the role of health workers in antenatal services is a good category. In a very small monetary sacrifice as much as 83.1% stated the role of health workers in antenatal services in the good category, and there were 16.9% included in the poor category.

Based on research results, they underestimate the problem of monetary sacrifice. because they say at the health center there is no charge if there is a BPJS and for those who do not have BPJS only the price of medicine is 10,000 and the average answer of 10,000 is not a burden for those who are important to get health services for their pregnancy well. And so is the transportation administration, most of them use ojeg to go to the health center at a price of 10,000, for those 10,000 for a motorcycle taxi at this time it is reasonable to return home from health center.

This study found that family income of pregnant women varies, family income greatly influences patient satisfaction with antenatal care, because if family income is high (51.8%) then the monetary sacrifice of pregnant women in obtaining antenatal care is not a problem, so in terms of monetary sacrifice it is categorized small. This research also found that husband jobs as a labor (35.5%), farmers (24.7%), entrepreneurs (29.1%), so they have enough money to get antenatal care.

This study is in line with the research of Lubis N & Martin (2009) which states that funding is not given much attention to the problem of getting optimal health services. Pregnant women are willing to pay more if health care providers improve the quality of services. Although the price is increasing every year, the patient is satisfied with the price of the room, the price of medicines. And others. This is because the higher price is also adjusted to the service so that patients get satisfaction and they are not price sensitive. People or the community will continue to use health services as long as price increases are also adjusted to the increase in services.[18,19,20,21]

3. Satisfaction of pregnant woman to the role of health workers in antenatal care based on the perception of sacrifice of time as an effort to reduce the risk of maternal death

Time sacrifice is the range of time that a customer must spend to obtain a product or service that they will use, or the amount of time that a customer needs to interact with a service company such as: whether fast or not is easy for customers to receive service from the company.[17]

Sacrifice time is the length of time sacrificed to get the service used to find out how much the cost of the customer to get the service.[21]

The results of research on the relationship between the role of health workers in antenatal care based on the perception of sacrifice of maternal time show the results of correlation analysis of 0.930 (p = 0.009) which means there is a correlation. The results of the study of assessing perceptions of time sacrifice based on the role of health workers in antenatal care show that at very large time sacrifices as much as 100%

stated the role of health workers in the antenatal service category is not good. In big time sacrifice as much as 90.2% stated the role of health workers in the antenatal service category is not good, while in small time sacrifice as much as 75.8% stated the role of health workers in antenatal service is a good category.

Based on research, the sacrifice of time felt by respondents is large, because sometimes respondents have to queue and wait for about 60 minutes or even more to get a pregnancy check-up service. but this time can be said that the time is not too long because the standard time set by the 2008 Ministry of Health Regulation is 60 minutes. However this is one of the reasons they state the role of health workers in pregnancy examinations at the health center is not good. This shows that the greater sacrifice of time is felt, it will give respondents a tendency to use the pregnancy check up less at the health center.

This study found that the education of pregnant women in middle school (38.7%), and high school (26.9%). So that their understanding of the importance of antenatal care is better. High education can provide opportunities to take advantage of antenatal care services, even though they have to sacrifice a lot of time.

To increase the perception of pregnant women regarding the quality of antenatal services based on time sacrifice, namely by making the small sacrifice of time felt by pregnant women by speeding up the time of service provided to pregnant women and all midwives in the MCH room should take their respective roles for serving pregnant women so that optimal service can be created and quickly resolved while reducing long queues.

This study is in line with the research of Muhammad Yusri (2015), Aulia Utami Dewi (2015) which states that there is a tendency that the fast waiting time for registration will make the patient satisfied with the service, or the length of the waiting time for registration will make the patient dissatisfied with the service and will have an impact on utilization return to the hospital.[22,23]

According to previous research, the relationship between waiting time of service and the level of patient satisfaction in the obstetric clinic and the content of Surakarta Hospital showed a positive relationship.[24] Researchers assume that the tendency of faster service waiting times increases patient satisfaction.[25,26]

In conclusion, satisfaction of pregnant women towards the role of health workers in antenatal care based on monetary sacrifice does not show correlation while based on time sacrifice shows there is a correlation. Need to increase the speed of the process of pregnancy examination services for pregnant women by health workers.

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Adolescents school students in Java and Sumatra are in greater risk of obesity

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Abstrak

Latar belakang: *Indonesia masih menghadai beban ganda masalah gizi berkaitan dengan obesitas yang meningat sementara masalah kurang gizi masih terjadi, termasuk pada remaja. Hasil penelitian masih terbatas, dalam hal aspek demografi dan geografi di Indonesia, sementara strategi pencegahan obesitas pada remaja membutuhkan intervensi yang lebih optimal. Tujuan: Studi ini bertujuan untuk memberikan gambaran masalah obesitas berdasarkan karakteristik populasi dan perilaku berisiko di region yang berbeda.*

Metode: *Studi ini menggunakan data sekunder dari survei kesehatan berbasis sekolah tahun 2015 yang dikembangkan oleh CDC Amerika dan WHO, dengan modifikasi sesuai kondisi Indonesia. Analisis mencakup 10,544 pelajar kelas 7 – 12 dengan representasi populasi nasional di tiga regional/pulau di Indonesia. Uji statistik yang digunakan adalah chi-square dan log regression.*

Hasil: *Model logistik menunjukkan pelajar remaja yang tinggal di pulau Jawa mempunyai risiko yang lebih tinggi untuk mengalami obesitas (adjusted OR 2.1;95%CI 1.3-3.3) dibandingkan pada pelajar yag tinggal di pulau Sumatra dan luar pulau Jawa dan Sumatra, sementara perilaku berisiko seperti aktivitas fisik dan perilaku diet tidak menunjukkan hubungan yang bermakna dengan kejadian obesitas.*

Kesimpulan: *Disparitas masalah obesitas terjadi pada remaja di tiga pulau besar di Indonesia, di tingkat kelas yang berbeda dan perilaku diet berisiko yang berbeda. Strategi pencegahan diperlukan lebih mengarah pada intervensi berbasis sekolah dengan memperhatikan faktor geografis tempat tinggal di pulau Sumatra dan lainnya serta tingkat atau kelas yang berbeda.* ***(Health Science Journal of Indonesia 2019;10(2):119-27)***

Kata kunci: *Obesitas, remaja, perilaku diet, region, aktivitas fisik*

Abstract

Background: Indonesia faces burden of nutrition related diseases as obesity is increasing while malnutrition still exists, including in adolescents. Research are limited in term of which specific demography and geography aspects in Indonesia while stronger strategic intervention to prevent obesity in adolescents is needed. **Objective:** This study aims to describe proportion of obesity in indifferent adolescents characteristic and eating behaviour in different regions.

Method: This study used data from Indonesia 2015 Global School-based Health Survey developed by US CDC and WHO) with modification based on Indonesia specific. The analysis included 10,544 students covered national representative and three regions of school students (grade 7 to 12) in Indonesia. Statistical analysis used chi square and log regressions.

Results: The logistic model showed adolescents students living in Java island has significantly higher risk of obesity (adjusted OR 2.1;95%CI 1.3-3.3) compare to their peers in outside Java and Sumatra Island, while behavior risk factors such as physical activity and dietary habit were not significantly associated with obesity.

Conclusions: Issues disparity of obesity in adolescents occurred in the three main Islands in Indonesia, in different school grades and in those with different dietary risk behaviours. Intervention strategy to address adolescents obesity issues will need to be directed toward school-based settings with taking into account specific approaches for students in Sumatra and other main islands in Indonesia as well as specific for junior and senior high school. ***(Health Science Journal of Indonesia 2019;10(2):119-27)***

Keywords: Obesity, adolescents, dietary behaviour, region, physical activity

Issues of obesity among adolescents have been rising recently in most of the world including less developed countries. Previously, a common assumption appeared that obesity occurred mostly in developed countries where high calorie food source and sedentary life style were very common even for the lowest income group. In fact, issues of obesity occurred within different perspectives between developed and less developed countries. A survey among adolescents in California showed that obesity prevalence significantly increased among lower income adolescents.[1] The prevalence of obesity was relatively low but increasing in less developed countries, including in Indonesia. In this case, non-communicable diseases are threatening and leading to social and economic impact of the population. Obesity adolescent increased the risk of certain obesity-related chronic diseases.[2]

In addition, several studies have been described that adolescents obesity contributed to higher academic and mental health problems such as lower self esteem, anxiety, depressive disorders and risk of suicide attempts.[3]

In terms of nutrition related issues, Indonesia still facing a double burden, where stunting is still high and obesity is on the rise. In general, factors related to obesity include low awareness of the harmful impact on obesity, stress-related eating, which lead to imbalance dietary intake or unhealthy diet during early age or infancy[4,5,6,7], as well as sedentary behaviours. Particularly for Indonesia, adult obesity related to post maternity period (for female) and cultural belief or value toward modern dietary lifestyle. Most of female obesity was related to short distance of child bearing period when dietary behaviour aimed to increase body weight for successful pregnancy which remained the same after the birth delivery due to unchanged dietary behaviours and most likely hormonal related contraceptive used. From the perspective of cultural factors, most people still believe that eating fast food, drinking soft drinks and other packaging drinks considered as 'modern' eating behaviour. Particularly for younger age groups, the high risk dietary habit along with parental eating behavior[8,9], as well as sedentary life style such as spent excessive time working on electronic devices such as TV, mobile phone or computers, may lead to adolescents of childhood obesity.

Obesity prevention may not effectively work using general single 'recipe'. It will requires adolescents specific strategies focusing on adolescent's characteristics and values related to eating behaviour, physical activities, and physical image. The perception of obesity causal factors are also different among those from lower and higher income populations, which may lead to the need for specific intervention for specific economic status subgroups.[10] Therefore it is necessary to study issues magnitude of adolescents obesity and it's characteristics and risk factors associated with adolescents. Specifically in Indonesia, adolescents characteristic dietary different may be varied across different islands, as Indonesia has seven main Islands. This paper aimed to describe behaviour risk determinants of obesity among adolescents. This paper aims to described behaviour risk deteriminants of obesity among adolescents, particularly in different social demographic and three different regions in Indonesia.

METHOD

Study design and population

A cross sectional study was carried out from January to November 2015 to provide accurate data on the proportion of sexual behaviour and its relationship with other health behaviours and protected factors among students. The 2015 Indonesia Global School-Based Health Survey (GSHS) is a school-based survey primarily for 12 to 17 years and conducted by the National Institute of Health Research and Development (NIHRD), Ministry of Health Indonesia. The GSHS was developed by WHO in collaboration with UNICEF, UNESCO, and UNAIDS, and with technical assistance from the US Centres for Disease Control and Prevention (CDC) Atlanta. Population in this survey comprised all junior and senior high school students (Year 7-12) across Indonesia with a total sample of 11,110 students.

The GSHS survey used a two-stage cluster sampling technique to generate a representative sample of students from class 7 to 12. In the first stage, CDC Atlanta selected a number of schools with probability proportional to school enrolment size using a specific computerised sample selection algorithm. Seventy-five schools spread across three regions (Sumatera, Java-Bali, and outer Sumatra and Java-Bali), 26 provinces and 68 districts were nominated. In the next stage, systematic sampling was employed to randomly select intact classrooms using a random start from each participated school. All classrooms within each selected school were included in the sampling frame, and all students in the sampled classrooms were eligible to participate in the study. Inclusion criteria in this study were all the students in grade 7 to 12 who registered in the selected class and schools and were attended at the school during the data collection. Exclusion criteria were those who were having illness and difficulty in response to the survey questions.

Data collection and variables

The 2015 Indonesia GSHS core questions include alcohol use, dietary behaviours, drug use, hygiene, mental health, physical activity, protective factors, sexual behaviours, tobacco use, violence and unintentional injury. Each core question consists of 3-7 questions. These core modules were used to address students' demographics, health behaviours and protective factors among students. The age variable is measured in years. In addition, the weights and heights were also measured among all students using standard portable electronic scales and stadiometers to collect information on the Body Mass Index (BMI). Obese was determined as had BMI > +2SD from median for BMI by age and sex.

In this study, Students completed the self-administered questionnaire during one class period between 20-30 minutes and record their responses directly in a computer-scannable answer sheet. The standardised instrument was used to collect the information on students' health-risk behaviours after being carefully adapted from the GSHS questionnaires. Prior to study, the questionnaires were initially translated into Bahasa Indonesia.

The variables used in this study are described in Table 1. The dependent variables were obesity, whereas independent variables included socio-demographics (age, gender, grade), behaviour risk factors such as physical inactivity and unhealthy diet. Detail explanation of each variable can be seen in table 1.

Data analysis

Data were coded and analysed using SPSS version 17. Descriptive analysis was done to obtain frequencies and proportions for the students' obesity status, socio-demographic characteristics and behaviour risk factors. Missing values were omitted during calculations of proportions. To assess the associations between obesity and all independent variables, bivariate and multivariate logistic regression were performed. In the bivariate analysis, a statistical significance was indicated from the P values less than 5%. The adjusted odds ratios (ORs) for the multistage stratified cluster sample design of the study, and two-sided 95% confidence intervals were accordingly reported. In the analysis, sample weights were also used to adjust for differences in the probability of selection between students.

Ethical consideration

Ethical approval was obtained from the National Ethics Commission on Health Research, National Institute of Health Research and Development Number LB.02.01/5.2/KE.158/2015. The survey put high concern on ethical aspects such as voluntary, confidentiality and knowledge based data utilization. Students were informed that they could withdraw from the study at any time before or during data collection and refuse to answer any questions, which they felt uncomfortable. To maintain confidentiality, no personal identifier was provided in the questionnaire and answer sheet.

Table 1. Variables related to the obesity in school-children based on GSHS, Indonesia, 2015

Variables	Questions	Response options
Obesity		
Height	The height measurement was conducted by trained survey administators using microtoise. The measurement result was reported by the students in the question: "How tall are you without your shoes on?"	Minimum recorded height:119 cm Maximumrecorded height:196 cm
Weight	The weight measurement was conducted by trained survey administators using weight scales. The measurement result was reported by the students in the question: "How much do you weigh without your shoes on?"	Minimum recorded weight: 22 kg Maximum recorded weight:45 kg
Socio-demographics		
Gender	"What is your sex?"	1 = Boy; 2 = Girl
Grade	"In what class are you?"	1 = *Kelas* 7 to 6 = *Kelas* 12 (coded 1 = 1 to 3; 2 = 4 to 6)
Dietary behavior		
Ate from fast food restaurant	"During the past 7 days, on how many days did you eat food from a fast food restaurant, such as KFC, McDonald, Texas Fried Chicken, California Fried Chicken, Burger King, or A and W?"	-
Usually drink carbonated drink	"During the past 30 days, how many times per day did you usually drink carbonated soft drinks, such as Coca-Cola, Sprite, Fanta, or Big Cola?"	1 = I did not drink carbonated soft drinks during the past 30 days to 7 = 5 or more times per day (coded 1 = 1 to 2; 2 = 3 to 7)
Consume vegetable	"During the past 30 days, how many times per day did you usually eat vegetable such as carrots, cabbage, spinach, or kangkong?	1 = did not eat vegetables; 2. Eat vegetable at least one or less.
Consume fruits	"During the past 30 days, how many times per day did you usually eat fruit such as pineapples, bananas, oranges or watermelons?	1 = did not eat fruits; 2. Eat vegetable at least one or less.
Physical activity		
Physically active	"During the past 7 days, on how many days were you physically active for a total of at least 60 minutes per day?"	1 = 0 days to 8 = 7 days (coded 1 = 6 to 8; 2 = 1 to 5)
Sit ≥3 hours per day	"How much time do you spend during a typical or usual day sitting and watching television, playing computer games, talking with friends, or doing other sitting activities, such as play station?"	1 = Less than 1 hour per day to 6 = more than 8 hours per day (coded 1 = 1 to 2; 2 = 3 to 6)

RESULTS

The total number of students who participated in this study was 10,544 students. This study showed the gender distribution of 48.9% males and 51.1% females. Most of the students in this study were in grade 7 to 9 (76.8%) or around the age of 13 to 15 years, while 12.23% is in grade 10 to 12.

The overall proportion of obese among the students was 5.2% whereas it was higher among males than females, in Java region and in grade 7 to 9. Adolescents characteristic distribution showed that proportion of obese was higher in those who practised unhealthy behaviour such as did not eat vegetables (7.1%), ate fast food once or more in a day (5.7%), sitting 3 hours or more per day (5.5%).

From Table 2, it is illustrated that proportion of obesity among those practiced unhealthy behaviours such as not physically active and consume unhealthy diet in the three regions, except for the region of Outside Java and Sumatra. In the region of outside Java and Sumatra the proportion of obesity and physical activity, showed uncommon results whereas the proportion of obesity was higher in those who were active (4.3% vs 2.9%). This may related to design bias whereas this study does not provide data on intensity and time components for causality relationship. The proportion of obesity in relation to unhealthy diet showed a similar pattern between the three regions. However, the pattern showed oppositely in java in terms of obesity and consumption of fast food.

Table 2. Proportion of obese by dietary behaviours and adolescents characteristics, Indonesia Global School-based Health Survey 2015

		P value	Proportion of Obese	95% Confidence Interval	
				Lower	Upper
Overall			5.2%	4.3%	6.2%
did not eat vegetables	yes	0.079	7.1%	3.7%	13.1%
	no		5.1%	4.3%	6.1%
did not eat fruits	yes	0.648	5.0%	3.5%	7.1%
	no		5.2%	4.3%	6.2%
ate from fast food restaurant one or more days	yes	0.011	5.7%	4.8%	6.8%
	no		4.6%	3.6%	6.0%
Physically active	yes	0.489	5.7%	4.1%	7.8%
	no		5.1%	4.3%	6.1%
usually drank carbonated drink	yes	0.194	5.0%	4.0%	6.2%
	no		5.3%	4.3%	6.5%
sitting >= 3 hrs per day	yes	0.076	5.5%	4.3%	7.0%
	no		5.1%	4.2%	6.1%
Sex	male	0.000	6.2%	5.0%	7.7%
	female		4.2%	3.6%	5.0%
School grade	grade 7 to 9	0.073	5.3%	4.3%	6.5%
	grade 10 to 12		4.8%	3.2%	7.0%
Region					
	Java	0.007	6.1%	4.8%	7.8%
	Sumatra		4.2%	3.2%	5.7%
	Other		3.0%	2.1%	4.3%

Table 3. Un-adjusted odds ratio of obese by dietary behaviours and physical activity stratified by regions, Indonesia Global School-based Health Survey 2015

	Proportion of Obesity								
	n	%	95% CI		n	OR	95% CI		P value
PA and/or sedentary									
Java									0.791
active	18	5.9%	3.7%	9.3%	267	.956	.625	1.462	
not active	212	6.1%	4.8%	7.8%	3349	Reff			
Sumatra									0.508
active	5	2.9%	1.1%	7.7%	156	.667	.222	2.004	
not active	139	4.3%	3.2%	5.8%	3233	Reff			
Outside Java and Sumatra									0.612
active	7	4.3%	2.1%	8.6%	197	1.479	.686	3.188	
not active	98	2.9%	2.0%	4.2%	3352	Reff			
unhealthy diet									
Java									0.451
no diet risk	75	5.8%	4.1%	8.1%	1262	.912	.680	1.224	
Have diet risk*	155	6.3%	4.9%	8.0%	2354	Reff			
Sumatra									0.729
no diet risk	56	4.1%	2.9%	5.9%	1376	.964	.720	1.290	
Have diet risk*	88	4.3%	3.2%	5.8%	2013	Reff			
Outside Java and Sumatra									0.839
no diet risk	39	3.0%	1.9%	4.7%	1360	.983	.672	1.439	
Have diet risk*	66	3.0%	2.1%	4.3%	2189	Reff			
fast food consumption									
Java									0.636
consume fast food >=1 per day	61	5.8%	4.0%	8.3%	1008	.925	.685	1.248	
Consume fast food <1 per day or none	169	6.2%	4.9%	7.9%	2608	Reff			
Sumatra									0.480
consume fast food >=1 per day	38	4.8%	3.2%	7.0%	811	1.186	.927	1.517	
Consume fast food <1 per day or none	106	4.1%	3.1%	5.3%	2578	Reff			
Outside Java and Sumatra									0.731
consume fast food >=1 per day	25	3.2%	2.0%	4.9%	796	1.075	.801	1.444	
Consume fast food <1 per day or none	80	3.0%	2.1%	4.2%	2753	Reff			

*soft drink >=1per day or fast food >=1 perday;

Table 4. Un-adjusted odds ratio of obese by dietary behaviours and physical activity stratified by grades, Indonesia Global School-based Health Survey 2015

	Proportion of Obesity								
	n	%	95% CI		n	OR	95% CI		P value
grade 10 to 12									0.535
active	16	7.2	4.1	12.2	224	.996	.547	1.814	
not active	168	7.2	5.0	10.2	2752	Reff			
grade 7 to 9									0.430
active	14	4.0	2.1	7.8	389	.948	.516	1.745	
not active	277	4.3	3.5	5.2	7147	Reff			
unhealthy diet									
grade 10 to 12									0.281
no diet risk	72	6.8	4.6	10.0	1278	.911	.609	1.364	
Have diet risk*	112	7.4	5.0	10.8	1698	Reff			
grade 7 to 9									0.430
no diet risk	98	4.0	2.9	5.4	2702	.894	.651	1.228	
Have diet risk	193	4.4	3.6	5.4	4834	Reff			
fast food consumption									
grade 10 to 12									0.394
consume fast food >=1 per day	47	7.6	5.0	11.3	684	1.081	.796	1.469	
Consume fast food <1 per day or none	137	7.0	5.0	9.9	2292	Reff			
grade 7 to 9									0.799
consume fast food >=1 per day	76	4.3	3.2	5.6	1920	1.014	.780	1.318	
Consume fast food <1 per day or none	215	4.2	3.4	5.3	5616	Reff			

*soft drink >=1per day or fast food >=1 perday;

Table 5. Adjusted odds ratio of obese by dietary behaviours and adolescents characteristic, Indonesia Global School-based Health Survey 2015

	Adjusted Odds Ratio	95% Confidence Interval		
		P value	Lower	Upper
PA and/or sedentary				
Active	1	0.846		
not active	1.035		.727	1.472
Sex		0.000		
Male	1			
Female	.944		.752	1.187
Region		0.007		
Outside Java and Sumatra	1			
Java	2.102		1.337	3.306
Sumatra	1.431		.886	2.311
unhealthy diet		0.961		
no diet risk	1			
Have diet risk*	1.072		.874	1.315

*soft drink >=1per day or fast food >=1 perday;

The association between obesity and behaviour risk factors was not showing significant and clear direction across different school grades. The pattern was similar in the two grades category (grade 10-12; grade 7 – 9). In Indonesia setting, grade 10-12 refers to senior high school and grade 7-9 refers to junior high school. The proportion of obesity was not showing significant different behaviour risk factors such as diet and physical activity across the different grade.

The adjusted odd ratio showed that adolescents students who stay in Java Island have significantly higher propotion of obesity after controlled by other indicatiors such as sex and behaviour risk factors of diet and physical activity (OR: 2. 1; CI:1.3-3.3).

DISCUSSIONS

The main findings of this study described that adolescents living in Java and Sumatra Island were actually leading to greater risk of obesity compared to other adolescents living in other Islands in Indonesia. Meanwhile, in addition to this fact, this study found indicators that show higher proportion of obesity, such as in those who did not eat vegatebles at least once a day.

Geographical determinant is most likely contributing to almost all health indicators in Indonesia in term of its association towards accessibility of sufficient quality health care delivery, communication technology available for adequate health education, and access to food. Indonesia is an island country that has seven main regions such as Sumatra, Java and Bali, Nusa Tenggara Barat, Nusa Tenggara Timur, Maluku - North Maluku, Sulawesi and Papua-Papua Barat. Population distribution is still an issue for Indonesia, as more than 80% of total Indonesian population reside in Java Island and country's development is more massively growing compare to other islands in Indonesia. Although, this condition also contributes to adolescents nutritional status including obesity.

It is indicated that the proportion of obesity among adolescents showed significantly higher in boys than in girls and boys. This finding was similar to the obesity study among adolescents aged 11-16 years in Canada on 2002 that showed the prevalence of obesity was 4.6% and it was higher in boys ($p < 0.00$) than girls[11] and also surveys in nine countries (Canada, Qatar, Taiwan, Cyprus, Czech Republic, Germany, Greece, Italy, Australia, Denmark, Hungary) showed prevalence of overweight among boys was ≥10% higher than girls.[12] On the contrary, some other countries, such in African countries, showed the prevalence of adolescents was higher in females than in males.[13]

Similar findings also found in other cross sectional study among adolescents aged 11-18 years in Southwest French, whereas prevalence of obese was higher among boys.[14] Another cross sectional study in Taiwan, that showed the prevalence of obese adolescents (13-16 years) was 7.2%.[15] The higher proportion of obesity in males compared to females adolescents was most likely related to diferent diet and physical activity patterns as well as physical maturation. Dietary pattern among male adolescents was more likely toward high fat and sugar dietary habit.[16] A survey in urban Saudi showed the

prevalence of obesity among adolescents was 24.1% in males and 14% in females adolescents aged 14 – 19 years. The gender different was mostly likely related to female has more concern on physical image that may lead to stronger dietary behaviour control in females.[17] Another study had shown that gender and obesity was related to different value, culture and stigma of obesity between male and female perspectives, whereas female put higher value of body image than male.[18]

Physical inactivity did not significantly related to adolescent obesity. The physical activity prevalence seems to be slightly different between male and female adolescents. A similar finding was found in a national survey in Lebanon, that showed no significant different obesity between active and less active adults.[19] Another study in South West French showed that overweight and obesity was significantly higher among adolescents with sedentary activity, (OR 1.33, 1.02–1.74, $P < 0.05$).[14] The relationship between obesity and physical inactivity may related to different physical maturation between males and females, which may lead to greater obesity risk on male adolescents than females. However, this study did not include indicator of physical maturation in the conceptual analysis as it is focused more on the behaviour risk indicators.

This study showed that insufficient intake of fruit and vegetable were not significantly related to obesity in adolescent while consumed fast food was significantly related to obesity. Similar result was found from a cross sectional study in Kajang Malaysia, that showed no significant different of body weight status and nutrition intake among adolescents[20] as well as a study in Australia[16], however other study showed that mediteranian diet that rich of fruits showed lowering risk of obesity.[21] Exessive food intake and lack of physical activity are the two main risks of obesity. Several studies have shown a relationship between diet and physical activity toward obesity.[22,23,11] A study among Jordanian adolescents showed that consumption of fried food and perceived stress level were positively correlated with overweight and obesity.[24]

However, as this study was limited to the fruit and vegetable intake pattern that was not including the quantity of the intake and other nutrient intakes such as fat, protein and carbohydrate food sources. Fruit and vegetable intake was fibre source foods that contribute to the macro nutrient absorption process in which sufficient and regular intake of them will bring positive to normal blood glucose and lipid profiles. This mechanism path was not directly related to obesity.

Fat intake was more likely directly associated with obesity due to its metabolism process and characteristic that allowed it to be stored in the form of fat tissue in the body. Consuming fast food one or more days per week in adolescents can be lead to higher risk of overweight and obesity among adolescents. A study in Iran also found that a higher intake of fast food lead to greater risk of overweight and obesity.[25] Higher food consumption is also known related to brain function reffered to executive disfunction that leads to the inability to control eating behaviours although further research is required for more detail causal relationship between executive function and obesity in adolescents.[26]

Consumption of fast food is one of common behaviours among adolescents in Indonesia as well as in many other countries as part of the modern lifestyle and food technology development. As one of the growing countries, Indonesia faces challenging issues in population health and environment along with the resources and infrastructure developments. National resources development such as in industrial sectors was influenced by the global development that also contributed to social and economic change in Indonesia. This study showed that similar pattern of relationship between fast food consumption and obesity in Sumatra island and outside Java and Sumatra, but not in Java where the proportion of obesity is higher in those who ate fast food less than one portion per day or none, although it is not significantly related. This finding may relate to other confounding factors that may occure in Java such as consumption of other high carbohydrate rich food that specifically common among certain culutural or tradition or ethnic.

National level policies and integrated efforts in food and nutrition have been developed in the form of a National Action Plan on Food and Nutrition 2015-2019. These policies and initiatives are typically designed to alter the food and physical activity environments to provide healthier choices for individuals within population.[27] School health activity or so-called *Upaya Kesehatan Sekolah* (UKS) is a program established by the government to provide support and motivation for children to implement a healthy life style as well as to provide healthy environment for the children.[28] Health

promotion initiatives therefore could be carried out in schools under the UKS program through integrated health education. Variety health education programs can be applied such as "Smart choices" and "Kitchen garden" as selection strategies for a healthy diet.[29]

On one hand, "Smart choices" program encourages schools to provide media such as posters about food and drinks categories. This program aims to educate children which food or drinks whether should be carefully consumed or should be eaten in large amounts. On the other hand, "Kitchen garden" program allows schools to provide small garden for children to plant vegetables, then cook them into healthy foods in the school kitchen.[29] Yet, these programs could not be properly carried out without supportive environment and adequate human resources and infrastructure. There is a need for high commitment between schools and other stakeholders to enforce health promotion initiatives among children so the prevalence of obesity in school children can be reduced as much as possible.

In conclusion, geographical determinant is an important component to develop a more specific intervention to prevent obesity among adolescents. Targetted adolescents in Java Island will bring wider impact on adolescents health, without ignoring specific needs from other regions.

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Correlation between dietary fat consumption with body mass index and body composition (a preliminary study in community based)

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Abstrak

Pendahuluan: *Sejumlah penelitian menunjukkan hubungan antara konsumsi lemak dengan indeks massa tubuh dan komposisi tubuh. Penelitian ini bertujuan untuk mengetahui hubungan antara konsumsi harian lemak total, asam lemak tak jenuh ganda (PUFA) dan kolesterol total dengan beberapa paramater gizi.*

Metode: *Penelitian ini merupakan studi cross-sectional, dengan 102 subjek. Pemeriksaan yang dilakukan adalah tinggi badan, berat badan, indeks massa tubuh dan pengukuran komposisi tubuh menggunakan timbangan komposisi tubuh Omron® HBF-212. Analisis konsumsi lemak total, asam lemak tak jenuh ganda dan kolesterol total menggunakan Software nutrisurvey 2007. Uji korelasi yang digunakan adalah Spearman Rho dengan menggunakan SpSS 21.*

Hasil: *Konsumsi lemak total tidak berhubungan dengan indeks massa tubuh, massa lemak total, dan massa lemak visceral. Konsumsi PUFA berhubungan secara negatif dengan indeks massa tubuh (p <0,014, -0,24) dan massa lemak total (p <0,001, -0,326), sedangkan konsumsi total kolesterol total berhubugan secara negatif dengan indeks massa tubuh (p <0,019, -0,23), dan massa lemak total (p <0,001, -0,337).*

Kesimpulan: *Ada hubungan antara konsumsi lemak dengan indeks massa tubuh dan komposisi tubuh.* ***(Health Science Journal of Indonesia 2019;10(2):128-31)***

Kata kunci: *konsumsi lemak; indeks massa tubuh; komposisi tubuh*

Abstract

Introduction: Studies showed some relation between fat consumption with body mass index and body composition. We conducted a study to investigate relationships between daily consumption of total fat, *polyunsaturated fatty acid* (PUFA) and total cholesterol with some nutritional parameters.

Methods: This was cross-sectional study, with 102 subjects. The study was examined height, body weight, body mass index and body composition measurements using the Omron® HBF-212 body composition monitor. There was nutrisurvey 2007 to measure total fat, polyunsaturated fatty acid and total cholesterol consumption. We analyzed the correlation by using SpSS 21 (*Spearman Rho*)

Results: Total fat consumption was not related to body mass index, total fat mass, and visceral fat mass. PUFA consumption was negatively associated with body mass index (p <0.014, -0.24) and total fat mass (p <0.001, -0.326), while consumption of total cholesterol was negatively associated with body mass index (p <0.019, -0.23), and total fat mass (p <0.001, -0.337)

Conclusion: There was a relation between fat consumption with body mass index and body composition. ***(Health Science Journal of Indonesia 2019;10(2):128-31)***

Keywords: fat consumption; body mass index; body composition

The quality of food intake can affect body composition. Consumption of macronutrients, especially fat, were associated with an increased of body fat mass which resulting in obesity. Obesity must be controlled because of their increased incidence both of developed or developing country, and they are related to the increased incidence of several comorbid diseases such as diabetes, cancer and cardiovascular disease.[1,2] Daily fat intake consists of several types, such as PUFA and cholesterol. Research showed that consumption of saturated fat was positively associated with total body fat ($p < 0.05$) and trunk fat ($p < 0.001$).[3,4] While research that shows the relationship between other types of fat intake has not been explored, especially in community based. This study aims to determine the relationship between consumption of total fat, PUFA and total cholesterol with nutritional status and body composition in community based.

METHODS

This was an observational study conducted in Universitas Sebelas Maret, using cross sectional design. It was held on March until September 2019. The subjects were 11 lectures, 20 administrative officers and 71 students of Medical Faculty Sebelas Maret University who met the criterias. All subjects had been given explanations and signed informed consent. We use a purposive sampling to selects 102 samples, the criteria were people aged above 17 years old and not use heart stent. Ethical clearance obtained from Ethics Committee of Universitas Sebelas Maret Number 363/UN27.06/KEPK/EC/2019.

The processed of collecting data was conduct by some trained enumerators. Height was measured by microtoise, while body weight, body mass index and body composition were measured using Omron® HBF-212 Body Composition Monitor. The classification of total fat and visceral fat mass level used its manual book. Total fat mass percentage was called 'low' if in the 5 to 9.9 range for man and 5 to 19.9 range for woman, it was called 'normal' if in the 10 to 19.9 range for man and 20 to 29.9 range for woman, it was called 'high' if in the 20 to 24.9 range for man and 30 to 34.9 range for woman, and it was called 'very high' if in the 25 to 50 range for man and 35 to 50 range for woman.Visceral fat mass percentage wasn't different between man and woman, it was called 'normal' if in the 1 to 9 range, and it was called 'high' if in the 10 to 14 range, and it was called 'very high' if in the 15 to 30 range.

Dietary fat intake was collected by interviewers using semiquantitative food frequency questionnaire. The results of the interviews were converted and analyzed with Nutrisurvey 2007 to determine the average intake of fats, PUFA and total cholesterol. Normality test was determined with Kolmogororv Smirnov that showed abnormal data distribution. The hypothesis was tested with Spearman Rho using SPSS v21 software and interpretate the correlation coefficients with Quinnipiac University's criteria.[5]

RESULTS

The subject characteristic of this study was showed in Table. 1. The statistical analysis of this study showed that total fat consumption was not related to body mass index, total fat mass, and visceral fat mass (Table. 2).

Table 1. Characteristic of subject

Characteristic	n (%)
Number of Subjects : 102	
Body Mass Index (BMI)	
- Underweight	13 (12,75)
- Normal	45 (44,12)
- Overweight	18 (17,65)
- Obese I	23 (22,55)
- Obese II	3 (2,94)
Sex	
- Male (♂)	26 (25,49)
- Female (♀)	76 (74,51)
Body Composition	
- Total Fat Mass (%)	
Low(♂:5-9.9; ♀:5-19.9)	5(4,90)
Normal (♂: 10-19.9;♀:20-29.9)	46(45,10)
High (♂: 20-24.9;♀:30-34.9)	27(26,47)
Very High (♂: 25-50;♀:35-50)	24(23,53)
- Visceral Fat	
Normal (♂ and♀: 1-9)	86(84,31)
High (♂ and♀: 10-14)	9(8,82)
Very High (♂ and♀: 15-30)	7(6,86)

PUFA consumption was negatively associated with body mass index ($p < 0.014$, -0.24) and total fat mass ($p < 0.001$, -0.326). The correlation coefficient of PUFA consumption and body mass index was -0.24, with a p-value of less than 0.014. This r of -0.24 is weak correlation, but has statistical significance.The correlation coefficient of PUFA consumption and total fat mass was -0.326, with a p-value of less than 0.001. This r of -0.326 is moderate correlation, and has statistical significance.

Consumption of total cholesterol was negatively associated with body mass index (p <0.019, -0.23), and total fat mass (p <0.001, -0.337). The correlation coefficient of total cholesterol consumption and body mass index was -0.23, with a p-value of less than 0.019. This r of -0.23 is weak correlation, but has statistical significance. The correlation coefficient of total cholesterol consumption and total fat mass was -0.337, with a p-value of less than 0.001. This r of -0.337 is moderate correlation, and has statistical significance. From food frequency questionnaire showed that the average total fat, PUFA and total cholesterol consumption in this study were 77 g, 4.5 g, and 283 g.

Table 2. Spearman Rho correlation coeffition between fat consumption, BMI and body composition

Variabel		Fat Consumption		
		Total Cholesterol	PUFA	Total Fat
BMI	Corr. Coeff.	-0.23	-0.24	-0,053
	Sig	0.019	0.014	0,596
Total Fat Mass	Corr. Coeff.	-0.337	-0.326	-0,01
	Sig.	0.001	0.001	0,921
Visceral Fat Mass	Corr. Coeff	-0,094	-0,118	0,097
	Sig.	0,345	0,237	0,33

DISCUSSION

This study analyzed the consumption of total fat, PUFA and total cholesterol. Total fat is one type of fat that cause some effect of their consumption on health. Maximum total fat consumption about 30% from total energy intake (TEI) could decrease the concentration of triacylglycerols (TAG), LDL-cholesterol, and increased concentration of HDL-cholesterol, and regulated insulin sensitivity.[6]

Classification of fatty acids according to their structure consist of saturated and unsaturated; saturated fatty acid (SFA), polyunsaturated and monounsaturated fatty acids. Both of them contain carbon chains varying between 2 and 36 carbon atoms. Polyunsaturated FA (PUFA) is characterized by pentadiene configuration of double bonds. This type is associated with protection from obesity-related phenotypes in adults. When someone had a higher intake of PUFAs and a higher ratio of PUFAs to SFAs are positively associated with lean mass and negatively associated with visceral adiposity and body fat in children.[7] The same result was also found in our study; the people who had a high BMI score, total fat mass and visceral fat mass would have low consumption of PUFA (Table 2). Our study showed that total fat consumption was not related to body mass index, total fat mass, and visceral fat mass (Table 2). It needs much long study to firm this conclusion. The result in high income countries, may not be applicable in low and middle countries.[8] There were Muka *et al*'s study, showed that no correlation between dietary fat composition with total fat mass and body fat distribution in women. It might be because of different fat metabolism in each gender; man or woman.[9,10]

This study also investigated of total cholesterol intake related to body composition. There're not much study tell, but one study showed a combined effect of saturated fat and cholesterol intake on serum lipids among Tehranian adults. The study showed that the intake of cholesterol and saturated fat have no combined effect on serum low-density lipoprotein cholesterol levels.[11] The study hypothesized that cholesterol consumption was atherogenic, and could increase cardiovascular disease risk.[12]

However, there is an evidence that saturated fatty acids and trans-fats increase cardiovascular disease risk. The fact that dietary cholesterol is common in foods that are high in saturated fatty acids might have contributed to the hypothesis that dietary cholesterol is atherogenic.[12]

The limitation of this study did not calculate the physical activity, other food intakes like sugar consumption, and method in assessing fat consumption; semiquantitative food frequency questionnaire. Studies that measure relation between physical activity and body composition showed that, greater physical activity was associated with lower average body fat percentage (for a BMI of 22.5-24.99 kg/m^2: 2.0 (95% CI 1.8 to 2.2), percentage points lower body fat in men and 1.8 (95% CI 1.6 to 2.0) percentage points lower body fat in women.[13] The study showed that consumption of dietary sugar could increase obesity and metabolic disease like type 2 diabetes[14], a further study that also calculates sugar intake are needed.

This study used semiquantitative food frequency questionnaire to collect fat consumption data. Although this method can give overestimated and underestimated result, it is easily used in epidemiology study, and have been validated both children or adult subjects.[15,16]

Similar studies can be enhanced by using larger

sample sizes, standardized body composition device based on dual energy x-ray absorptiometry (DEXA), combined methods in collecting and assessing historical diet, combined method in analyze and count the fat intake, and also calculate other factors that influence body composition.

In conclusion, there was a correlation between fat consumption with body mass index and body composition. PUFA consumption was negatively associated with body mass index and total fat mass, while consumption of total cholesterol was negatively associated with body mass index and total fat mass.

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Profile of malondialdehyde (MDA) and catalase specific activity in plasma of elderly woman

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Abstrak

Latar belakang: *Malondialdehida (MDA) merupakan petanda stres oksidatif yang merupakan produk akhir dari reaksi berantai proksidasi lipid. Untuk mencegah stres oksidatif, tubuh mensintesis katalase, suatu enzim antioksidan endogen yang mengkatalisis hidrogen peroksida (H_2O_2) menjadi air dan oksigen. Sampai saat ini kadar MDA dan katalase pada populasi usia lanjut (usila) masih memberikan hasil yang bervariasi dan kadar tersebut pada kelompok usia yang berbeda dalam populasi usila belum pernah dilaporkan. Dengan demikian, penelitian ini bertujuan untuk menganalisis profil kadar MDA dan aktivitas spesifik katalase pada plasma populasi usila berdasarkan peningkatan usia.*

Metode: *Penelitian ini menggunakan 60 subjek wanita usila sehat yang tinggal di Jakarta. Subjek dibagi dalam 2 kelompok berdasarkan kategori usia, kelompok yang lebih muda (60 – 70 tahun) dan kelompok yang lebih tua (> 70 tahun). Kadar MDA dan aktivitas spesifik katalase dianalisis pada plasma dengan spektrofotometer.*

Hasil: *Kadar MDA pada kelompok yang lebih muda (60 – 70 tahun) sedikit lebih tinggi dibandingkan kelompok yang lebih tua (> 70 tahun) namun tidak bermakna secara statistik. Selain itu, akitivitas spesifik katalase pada kelompok yang lebih muda lebih rendah bermakna dibandingkan dengan kelompok yang lebih tua.*

Kesimpulan: *Tidak ada perbedaan bermakna kadar MDA plasma pada populasi usila. Namun, aktivitas spesifik katalase meningkat bermakna seiring dengan pertambahan usia.* ***(Health Science Journal of Indonesia 2019;10(2):132-6)***

Kata kunci: *Malondialdehida, katalase, wanita usila*

Abstract

Background: Malondialdehyde (MDA) is a marker of oxidative stress as an end product from the chain reaction of lipid peroxidation. In order to prevent oxidative stress, our body synthesizes catalase, an endogenous antioxidant enzyme that catalyzes hydrogen peroxide (H_2O_2) into water and oxygen. Until now, the level of MDA and catalase in aging population were still varied and those level at different age in elderly population has not been yet reported. Therefore, the purpose of this study was to analyse the profile of MDA level and catalase specific activity in plasma of elderly women based on increasing age.

Methods: This research used 60 healthy elderly women as the subjects living in Jakarta. The subjects were divided into 2 groups based on age category, the younger group (60 – 70 years old) and the older group (>70 years old). MDA and specific activity of catalase were analyzed in plasma using spectrophotometer.

Results: MDA level in the younger group (60-70 years old) was slightly higher than MDA levels in the older group (>70 years old) but it was not significant. Moreover, specific activity of catalase in the younger group was significantly lower than the older group.

Conclusions: There was no difference in MDA level of elderly woman between younger and older group. However, catalase specific activity significantly increased with increasing age. ***(Health Science Journal of Indonesia 2019;10(2):132-6)***

Keywords: Malondialdehyde, Catalase, elderly woman

Aging is a multidimensional process, in which the mechanism of destruction and repair in the body or the system occurs alternately at different speeds and times.[1] The aging process is difficult to understand because it is also difficult to distinguish between the normal aging process and the process due to an illness.[1] One factor as the contributor in aging process is oxidative stress which occurs due to excessive production of reactive oxygen species (ROS) exceed antioxidant capacity.[2] ROS is produced regularly from cellular respiration in mitochondria, phagocytosis process and hydroxylation of drug in liver. Moreover, environment pollution and radiation contamination exposed in the body act as external source of ROS.[3] Excessive ROS can irreversibly damage cellular components and cause cell death through the intrinsic apoptosis pathway in the mitochondria and triggers mitochondrial DNA damage.[4] Increased apoptosis is associated with cell reshuffle and telomere shortening at the ends of the DNA which limits the amount of cell mitosis. The increase in the number of telomeres lost due to imbalance in ROS production is one of the factors in the aging process.[5] ROS is very reactive and destructs various biomolecule around it such as protein, deoxyribose nucleic acid (DNA) and lipid especially polyunsaturated fatty acids (PUFAs).[6] Oxidation of PUFA form malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE) and other products such as *F2-isoprostanes*. MDA, HNE and *F2-isoprostanes* are oxidative stress biomarker which is widely used to detect lipid peroxidation.[6] MDA is highly reactive compound, easily penetrate into the tissues and able to form covalent bond with protein and nucleic acid allowing modification its structure and function.[7] The process caused loss of cell membrane integrity which can subsequently lead to disruption of cell function and ultimately cause dysfunction of individual organs.[7]

In order to overcome oxidative stress, the body synthesizes an endogenous antioxidant such as catalase.[8] Catalase is an antioxidant enzyme found in almost all living organisms that catalyze the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen.[8] Hydrogen peroxide (H_2O_2) is produced during cellular respiration in all living cells.[3] H_2O_2 is dangerous and must be disposed of as soon as possible. The cells containing small amount of catalase are very susceptible to be oxidized by H_2O_2. Therefore catalase plays an important role in the cell's defense mechanism against the oxidation attack of H_2O_2.[8] Until now, the researches about MDA and catalase level in aging population especially in Indonesia is still limited. MDA level in 41 elderly subjects age 60 – 90 years old at Social Rehabilitation *Unit Pucang Gading Semarang* was 12.69 ± 1.373 nmol/mL.[9] However, no report about catalase level in those population. In India, Akila et al[10] found that MDA level in 13 subjects of elderly age 60- 75 years old was 3.96 ± 43.58 nmol/mL, while catalase level was 48.03 ± 24.002 Unit per gram hemoglobin. They found that MDA level increased while catalase decreased in elderly compared to 15 subjects of normal young age 20 - 32 years old. Most of research compare oxidative stress between elderly and adult subjects, however there is no research which elaborates oxidative stress level at different age in elderly population. Therefore, the purpose of this study was to analyse the profile of plasma malondialdehyde (MDA) level and specific activity of catalase in elderly women based on increasing age. The elderly women used in this research because the number of elderly women in Indonesia (9,53%) are greater than the number of elderly men (8,54%) according to Data and Information Center (2017), Ministry of Health, Republic of Indonesia.

METHODS

It was a cross sectional research using 60 subjects of healthy elderly women aged 60 years and over who live in Kali Anyar, Tambora, West Jakarta.This location was selected because it is a slum and populous urban area thus the exposure to free radical was probably high. The women were chosen as the research subjects due to life span of women is longer than men, therefore this study might be able to provide an overview of oxidative stress profile in longer life span population who live in slum urban area, The subjects were divided into 2 groups based on age category, 30 subjects of the younger group (60 – 70 years old) and 30 subjects of the older group (>70 years old). The inclusion criteria in this study were women aged 60 years and over who were willing to be the subject. While, the exclusion criteria were the subjects with total immobility, acute phases of diseases such as respiratory infections (such as pneumonia), acute arthritis, stroke, coronary heart disease, hypertensive emergencies/ urgency as well as acute exacerbation of chronic obstructive pulmonary disease (COPD). Whole blood was taken from each subject and then centrifuged at 3000 rpm for 15 minutes to obtain plasma for MDA and catalase assay. All procedures have been approved by the Ethical Committee of Faculty of Medicine, Universitas Indonesia number 0910/UN2.F1/ETIK/2018.

MDA level

MDA assay was analyzed in plasma using spectrophotometer by thiobarbituric acid method.[11] Two hundred microliters of Trichloroacetic acid (TCA) were added to the sample and centrifuged at 5000 rpm for 10 minutes, pellet discarded and 0,4 mL of thiobarbituric acid (TBA) reagent was added. The solution was incubated in a boiling water bath for 10 min to produce pink color. After cooling at room temperature, samples were read at 532 nm using a spectrophotometer.

Specific Activity of Catalase Enzymes

One hundred microliters of the sample were added to 1,900 µl of H_2O_2 with optimal dilution. And then 100 µl of solvent was added following by homogenization with manual shaking and its absorption measured at 210 nm.[12]

Statistical analysis

Statistical analysis was performed using Statistical Package for Social Science (SPSS) software for Windows version 2.0. All data were tested for normality using the Kolmogorov-Smirnov Test. Significance test used Mann Whitney (nonparametric) for MDA level due to abnormal distribution and unpaired (parametric) t test for catalase.

RESULTS

MDA Level

The MDA level in 30 subjects of younger group (60-70 years old) was 0,039-3,826 nmol/mL, median 2,07 nmol/mL. While the MDA level in the 30 subjects of older group (> 70 years old) was 0,163-6,079 nmol/mL, median 1,93 nmol/mL (figure 1). There was no statistically different between those groups (p>0,05).

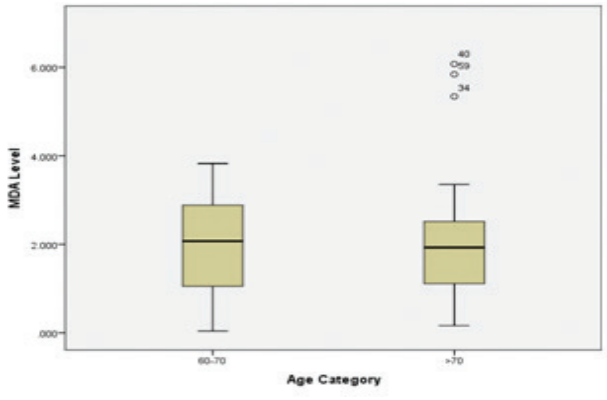

Figure 1. The plasma MDA level in the elderly 60-70 years and the elderly >70 years old. There was no significant differences (p> 0.05).

Specific Activity of Catalase Enzymes

In this study, the specific activity of the catalase enzyme in younger group (60-70 years old) was 0.047 ± 0.006 U/mg protein and 0.060 ± 0.004 U/mg in the older group (> 70 years old). From that results it meant that catalase specific activities of the older group was higher than the younger group (figure 2). That difference was stastically significant (p = 0.007).

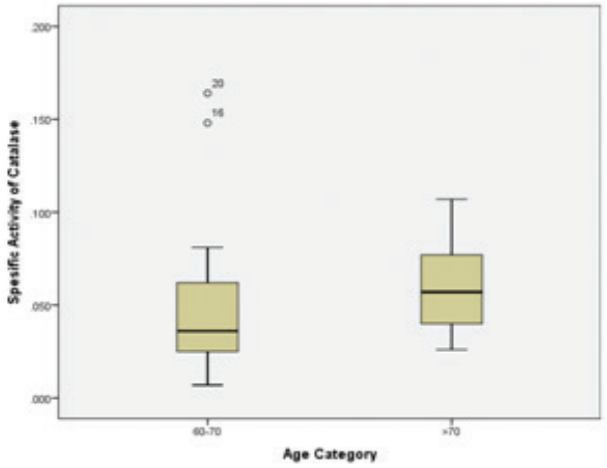

Figure 2. The specific activity of the catalase enzyme in the older group (> 70 years old) was significantly higher (p <0.05) compare to younger group (60-70 years old).

The MDA and catalase levels were also classified based on 5 years of age distance as shown in table1. MDA level tends to increased up to 69 years old. However, its level tends to decrease up to 84 years old. While in catalase, the level was gradually increased up to 84 years old. The results in this category could not be analyzed statistically since the number of samples in each category were small and not equal.

Table 1. MDA dan catalase level in women elderly based on 5 years age distance category

Age (years)	n	MDA Level Median* (min ± max)	Catalase level Mean ± SD
60-64	17	1.942 nmol/mL (0,039 ± 3,721)	0,043 ± 0,032 U/mg protein
65-69	9	2.570 nmol/mL (0,791 ± 3,826)	0,054 ± 0,047 U/mg protein
70-74	16	2.099 nmol/mL (0,215 ± 6,076)	0,055 ± 0,022 U/mg protein
75-79	13	1.680 nmol/mL (0,163 ± 3,250)	0,057 ± 0,025 U/mg protein
80-84	4	1.522 nmol/mL (1,114 ± 5,884)	0,076 ± 0,025 U/mg protein
100	1	1.929 nmol/mL	0.060 U/mg protein
	60		

Note: *Median was represented for MDA level due to abnormal distribution

DISCUSSIONS

Oxidative stress is a condition that reflects an imbalance between reactive oxygen species (ROS) and antioxidant defenses.[8] Malondialdehyde (MDA) is a marker of oxidative stress as the end result of a chain reaction of lipid peroxidation.[6] In this study, MDA levels of the elderly women in 60-70 years old group was 0,039-3,826 nmol/mL, median 2,07 nmol/mL. While MDA level in the > 70 years old group was 0,163-6,079 nmol/mL, median 1,93 nmol/mL Statistical tests showed no significant differences in the MDA levels between those groups ($p > 0.05$). The age range of research subjects was too close so that MDA levels between those groups were not significantly different. If we compared it with the MDA level in 10 young women (20 – 27 years old) that we checked (data not shown), MDA level in those elderly women was higher than young women (1,155 nmol/mL). When the subjects were grouped in 5 years distance as shown in table 1, actually MDA level tends to increase up to 69 years old and the highest MDA level was found in group 65 – 69 years old. Therefore in this age group, the women elderly probable were prone to suffer from degenerative diseases. In general, other studies compared MDA level between young and old age with a wide age range, such as Fasna et al[13] analyzed MDA levels in 150 healthy men and women aged between 20 and 90 years old. They found that plasma MDA levels increased with age, indicated rapid oxidation occurred during aging process.[13] Moreover, the increased of MDA level probably due to reduced antioxidants in the body. Muralidharan et al[14] proved that a decrease in the antioxidant level causes an increase in MDA level in the elderly population. It is known that the body continuously produces free radicals, both through normal metabolism, inflammation, malnutrition and environmental effects such as pollution, ultraviolet, cigarette smoke and others.[3] The formation of free radical compounds is the initiator of the lipid peroxidation process or MDA formation which acts as a destroyer of body tissue.[6] Therefore, as we get older, the buildup of free radicals increased in the body, resulting in oxidative stress. Currently MDA is more often used in biomedical research as the marker of oxidative stress especially in various clinical conditions related to the lipid peroxidation process. The more chemically stable properties of MDA make this compound more often used as the marker of oxidative stress.[7]

From the results of this study it was found that the specific activity of the catalase enzyme was significantly higher at the older group (> 70 years old) compared to the younger group (60-70 years old). If the subjects were groups in 5 years distance as shown in table 1, specific activity of catalase enzyme tends to increase with increasing age. It might be a protective effect of healthy elderly women in order to cope high level of oxidative damage. The function of endogenous antioxidant catalase is to suppress oxidative damage by catalyzing the change of H_2O_2 into water and oxygen.[8] High reactive free radicals could attack the cell membrane, which triggers high catalase activity in an effort to suppress the presence of oxidative stress.[6] The high specific activity of the catalase enzyme in this study seems to provide a protection thereby plasma MDA levels in this study did not increase with age. Several studies stated that free radicals are the main cause of aging.[2,15-17] Therefore, it is important to control the formation of free radicals by improving cellular antioxidant status to inhibit aging process. Until now the relationship between endogenous antioxidant activity and aging still needs to be elaborated more deeply, but it is often assumed that antioxidants serve as an anti-aging molecule.[18] All elderly subjects in this research were in healthy condition, due to catalase specific activity increased significantly in the older groups, it can be assumed that to achieve healthy aging conditions, endogenous antioxidants should be maintained at the proper level. Further study is needed to determine the value limit for endogenous antioxidant level which can be used as the standard for healthy aging status.

In conclusion, there was no difference in MDA level of elderly woman between younger (60 – 70 years old) and older group (>70 years old). However, catalase specific activity significantly increased with increasing age.

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